GLYCOPEPTIDES FROM GAMMA GLOBULINS

by

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I. INTRODUCTION

The γ-globulins have been the object of considerable study since it was learned that the γ-globulin fraction embodies most of the antibody activity of plasma proteins. In fact, it has been suggested that all γ-globulins are antibodies (1). It is well established that reagins and the classic antibodies, such as hemolysins and other lysing antibodies, precipitins, agglutinins, antitoxins and antibody activities which are manifested in compliment fixation and anaphylaxis are found in the γ-globulin fraction, and, moreover, these various activities appear to be carried by the same molecules (1). This is not to say, however, that these activities are associated only with γ-globulin; antibodies have been found in the α- and β-globulin fractions in some instances. Hyper-immune equine sera to tetanus, for example, have been found to contain the antibody in the β- and γ-globulin fractions, and chicken antibody to human γ-globulin was found in the α- as well as the γ-fraction (2). The substances responsible for some types of immune activity, such as the phenomena of delayed sensitivity and graft rejection, are not known (1).

The γ-globulin fraction has been shown to be heterogeneous by a number of criteria, including electrophoretic, sedimentation, chromatographic, solubility and immunochemical properties. The properties of γ-globulins and antibodies have been reviewed extensively (3-5).

γ-Globulins are arbitrarily defined as the group of proteins which move most slowly when serum is submitted to electrophoresis at pH 8.5 to 8.6. The conditions of electrophoresis suggested by Longworth, i.e., pH 8.5 to 8.6 barbiturate (Veronal) buffer with an ionic strength of 0.1, are most commonly used (1,2). The proteins of the γ-globulin fraction have a rather wide range of mobilities under these conditions. Deutsch et al. showed that normal human γ-globulin could be separated into two major fractions under these conditions. The faster migrating fraction is called γ_1-globulin, while the larger, slower migrating fraction is referred to as γ_2-globulin. This nomenclature has been extended to other species. γ_1-Globulin is also referred to as T-globulin (1,2). An even greater degree of heterogeneity in the γ-globulin fraction has been demonstrated by the technique of immunoelectrophoresis (1,6). The isoelectric points of the γ-globulins generally fall in the range of approximately 5.6 to 7.6, but lower values down to 4.4 have been reported for some antibodies, notably horse, cow and pig antipneumococcal antibodies (1).

In all species investigated, the main component of γ-globulin has a sedimentation coefficient of about 7 S. The molecular weights reported for this component by a number of investigators fall within the range of 150,000 to 190,000 (1,7). The differences in the values obtained cannot be correlated with the species nor with a difference in proteins within the 7 S fraction in a given species, and it appears that the observed differences are probably due to experimental error (1). In addition, a second, minor component with a sedimentation coefficient of approximately 20 S and a molecular weight between 900,000 and 1,000,000 has been found

in most normal sera examined. These macroglobulins have been variously reported to comprise 1 to 3% (1) and 5 to 10% (7,8) of the total γ-globulin, while the remainder is largely accounted for by the 7 S component. It has been suggested that these macroglobulins arise through the polymerization of the 7 S components. While this is known to occur, particularly when γ-globulin is isolated by cold ethanol precipitation, the fact that macroglobulins containing a considerably greater percentage of carbohydrate than the lower molecular weight species have been found in γ-globulin seems to leave little doubt that at least part of the macroglobulins are not artifacts. The 7 S component of normal human γ-globulin, for example, contains about 3% carbohydrate, whereas the 20 S component contains approximately 10% carbohydrate (8,9). The majority of the macroglobulins migrate in the γ_1 region (8). In addition to the macroglobulins normally present in γ-globulin, certain antibodies, including antipneumococcal antibodies in the horse, cow and pig, have molecular weights between 900,000 and 1,000,000. As noted above, these antipneumococcal antibodies have unusually low isoelectric points. In the rabbit the antipneumococcal antibodies are of the 7 S variety (1).

The heterogeneity of γ-globulin has also been revealed by chromatographic studies. Peterson and Sober and coworkers (10-12) have studied the chromatographic behavior of serum proteins on ion-exchange cellulose columns. When human and equine sera were chromatographed on diethylaminoethyl (DEAE) cellulose under the chromatographic conditions originally adopted for fractionation of serum proteins (12), most of the γ-globulin emerged before the other serum proteins as two or three incompletely separated large peaks followed by several smaller peaks; the former

corresponded in electrophoretic mobility to γ_2-globulin and the latter to the faster migrating components of γ-globulin. A small amount of γ-globulin emerged later with other serum fractions. By chromatography on DEAE cellulose of normal human γ-globulin isolated by zone electrophoresis techniques, Fahey and Horbett (13) have separated this γ-globulin into five fractions. The first to emerge from the column (component 1), which comprised about 71% of the total γ-globulin, and components 2 (7.7%), 3 (9.7%) and 4 (3.9%) were all 7 S components; component 5 (7.7%), the last to emerge, was mainly 19 S with a trace of 7 S component. The hexose contents of components 1, 2, 3 and 4 were reported as 1.1, 1.6, 1.9 and 2.3%, respectively; that of component 5, in good agreement with the known hexose content of normally occurring macroglobulin (9), was 5.0%. Thus, it appears that not only the macroglobulins but also minor 7 S components of normal human γ-globulin may contain more carbohydrate than the major 7 S portion. The electrophoretic mobilities of the individual components all fell within the γ-globulin range and increased in the same order as the hexose contents up to component 4; the mobility of the macroglobulin fraction was between the latter and the major, slowest moving fraction.

γ-Globulin may be separated from other serum proteins and divided into subfractions by precipitation methods, which include salting out and isoelectric precipitation techniques and separation on the basis of differences in solubility in water and in organic solvents (14). γ-Globulin may be separated into a euglobulin (water-insoluble) fraction and a pseudoglobulin (water-soluble) fraction simply by dialyzing against water and collecting the resultant euglobulin precipitate. Cohn *et al.* (15,16) have developed a procedure for the fractionation of serum proteins using

ethanol at low temperatures with controlled ionic strength, pH and protein concentration. Fraction II of Cohn is mainly γ_2-globulin; fraction III contains mainly γ_1- and β-globulins (2,14). Oncley et al. (17) have modified and extended the fractionation procedure of Cohn and coworkers and have separated γ-globulin into subfractions by these techniques.

In a number of instances specific antibodies have been identified with particular subfractions of γ-globulin. Antipneumococcal antibodies in the horse, cow and pig, for example, are largely associated with T-globulin, and, as previously mentioned, these same antibodies are in the macroglobulin component of γ-globulin. The corresponding antibodies in rabbit are associated with γ-globulin of lower mobility and are 7 S components (1). The isoagglutinins of normal sera are associated with γ_1- and β-globulins; these also belong mainly to the macroglobulin class, although smaller molecular species of isoagglutinins have been reported (1). Separation of antibody activity to various antigens has been achieved by means of ion-exchange chromatography by Fahey (18), Peterson and Sober, and others (10). Porter (1) has achieved fractionation of γ-globulin by partition chromatography on kieselguhr columns and observed that antibodies from rabbits immunized to a variety of antigens appear first in the slower moving fractions, then in both the slower and middle fractions, and, finally, almost entirely in the middle fraction. Changes in the electrophoretic mobility of antibodies after their initial appearance in the blood have also been observed (1). Enrichment of antibody activity in γ-globulin by fractional precipitation methods has also been reported (2); certain horse antibodies, for example, are concentrated in the pseudoglobulin fraction of γ-globulin, whereas others are predominately euglobulins.

The studies related above indicate, as has been suggested (2), that certain antibodies may be associated with a particular subfraction of γ-globulin.

In contrast to the differences in their physical properties, previous studies have shown that γ-globulins from a given species demonstrate a high degree of homogeneity with respect to their antigenic properties. γ-Globulins of different electrophoretic mobilities are antigenically similar, though slight differences have been observed in some instances (1,5). Horse macroglobulin antibody has been found to be antigenically different from normal γ-globulin (1). Four 7 S components isolated from normal human γ-globulin by Fahey and Horbett (13), as described above, were antigenically indistinguishable by gel diffusion tests. These components, however, could be distinguished by this method from a fifth, macroglobulin component. These authors concluded that γ-globulins possess at least two antigenic properties, one characteristic of the 7 S components and the other characteristic of the macroglobulins. Other recent findings (1) indicate that γ-globulin from one rabbit may be antigenic in another rabbit; several types of γ-globulins, called "allotypes," were identified by precipitation bands in gelatin. Thus, it would appear that γ-globulin is heterogeneous with respect to immunochemical as well as various physical properties.

In view of the heterogeneity of γ-globulin, as demonstrated by these criteria, chemical studies on γ-globulin would scarcely seem to be warranted. However, the chemical compositions of γ-globulins from various mammalian species and of subfractions of γ-globulin within a given species have been shown to be very similar, and, in some instances,

the subfractions appear to have identical compositions. Because of the physical heterogeneity of γ-globulins, a consideration of the method of isolation takes on added importance in comparing the chemical compositions of γ-globulins and subfractions of γ-globulins.

Smith et al. (19) have performed complete amino acid analyses on four different rabbit antipneumococcal antibodies, which were isolated by precipitation with the specific antigens. The results show that, within experimental error, there are no differences in the amino acid compositions of the antibodies to these closely related polysaccharide antigens. A comparison of the amino acid composition of these antibodies with that of normal human γ-globulin isolated by cold ethanol precipitation and analyzed by Brand et al. (1) shows that, although there appear to be some differences, the amino acid compositions of these γ-globulins closely parallel each other. Smith and coworkers (2) have also compared the amino acid compositions of human γ-globulin and subfractions of human γ-globulins isolated by ethanol precipitation techniques and those of electrophoretic fractions (γ_1 and γ_2) of bovine and equine γ-globulin. These analyses, though not complete, show a close relationship between the γ-globulins of these species. There are, however, what appear to be significant differences among the species and between different fractions within the same species. Fractions of the same electrophoretic mobility from normal and hyperimmune animals appear not to differ in chemical composition. In making such comparisons, however, it should be kept in mind that, with proteins of such size, a difference of a few tenths of a per cent of any one amino acid would be equivalent to several residues of the amino acid.

Amino-terminal amino acid analyses have been performed on γ-globulins from several species (1). Normal human γ-globulin and the II-3 fraction of human γ-globulin are reported to have one amino-terminal aspartyl and one amino-terminal glutamyl residue. The II-1,2 fraction of human γ-globulin, on the other hand, has been reported by two groups of investigators to have two rather than one amino-terminal glutamyl residue in addition to the terminal aspartyl residue. Small quantities of other amino-terminal amino acids were detected in all fractions. It is possible that the additional amino-terminal glutamyl residue in the II-1,2 fraction is an artifact.

A more complex picture is presented by horse and bovine γ-globulin (1), in which several amino-terminal amino acids have been detected, none of which was present in molar ratios. The γ- and β-globulins of the horse and cow are not separated as well under standard conditions of electrophoresis as are those of the human and rabbit, and it has been suggested that part of the γ-globulin fraction of these species is β-globulin of the same mobility. This is supported by the work of McFadden and Smith (20), who found a larger amount of amino-terminal valine in γ-globulin from a hyperimmune cow. This suggests that the true γ-globulin has a single amino-terminal valyl residue. By partition chromatography, Porter (1) has fractionated bovine γ-globulin into fractions rich in amino-terminal valine and others in which other amino-terminal amino acids are concentrated.

Rabbit γ-globulin is much less complex in that it contains only one amino-terminal alanyl residue per molecule and smaller amounts of amino-terminal aspartic acid, ranging from about 0.1 to 0.4 of a residue (1).

Normal rabbit γ-globulin, antiovalbumin, antibovine serum albumin and 8 antipneumococcal antibodies have been found to have amino-terminal alanine (1,21). Porter (22) has demonstrated that normal rabbit γ-globulin and antiovalbumin are alike in possessing the amino-terminal tetrapeptide sequence alanylleucylvalylaspartyl, with a glutamyl residue probably in the fifth position. McFadden and Smith (21) found the same amino-terminal pentapeptide sequence in four rabbit antipneumococcal antibodies. These same antipneumococcal antibodies were reported by Smith et al. (19) to have the same amino acid composition, within experimental error. Based on these findings, it has been suggested (1,21) that an identical amino acid sequence is probably present in a considerable part of the peptide chain of rabbit γ-globulins.

Various explanations have been advanced to account for the observed differences with respect to the physical properties of γ-globulin and, at the same time, account for the high degree of similarity with respect to chemical composition and, to the extent it is known, primary structure. The γ-globulins have their origin in the reticulo-endothelial system, which is concentrated in the spleen, lymph nodes, bone marrow and lungs (23), and it has been suggested (1) that these various tissues, or different cells within the same tissue, may produce slightly different types of γ-globulin. This possible explanation has some experimental support. Porter and coworkers (1) have been able to fractionate γ-globulin according to tissue origin by partition chromatography. In vitro studies of the rates of incorporation of radioactive amino acids into γ-globulin showed that the spleen labeled the middle fraction most rapidly, whereas lymph nodes and, to a lesser extent, the bone marrow labeled the slower

fractions most rapidly. It was concluded that different tissues synthesized different fractions at varying rates and that this might arise because the fractions were synthesized by a variety of different cells which are unevenly distributed among the tissues.

Pauling (24) has proposed that γ-globulins differ only in configuration. According to this idea, γ-globulin has a labile structure which gives rise to various configurations with the majority of the molecules assuming the most stable configuration.

A third proposal (1) is that the observed differences in γ-globulins are due to changes in environment in the tissues responsible for γ-globulin synthesis. This seems to be related to the template theory of γ-globulin synthesis (1), which would explain the specificity of antibodies by having the antigen alter the γ-globulin during synthesis in such a way that the γ-globulin is adapted complimentarily to the antigen.

As previously indicated, the γ-globulins, like many of the plasma proteins, are glycoproteins. Although a great deal of research, especially in recent years, has been directed toward gaining an understanding of the nature of glycoproteins and other protein-carbohydrate complexes, present day knowledge of the chemical nature of these substances is limited mainly to how much and what kind of carbohydrate components they contain; in some instances, however, more detailed knowledge has been attained. Likewise, little is known of the physiological role of protein-carbohydrate complexes. Glycoproteins and other protein-carbohydrate complexes have been treated in a number of reviews (9,25-27).

Six types of monosaccharide are recognized as being regular components of plasma proteins; these are galactose, mannose, glucosamine,

galactosamine, fucose and sialic acid. In normal plasma, the ratio of glucosamine to galactosamine is about 10 to 1, and only a single type of hexosamine has been found in any one glycoprotein (9). Human plasma proteins contain only N-acetylneuraminic acid (O-sialic acid), while the plasma proteins of some animal species contain N-glycolylneuraminic acid (P-sialic acid) in addition to the N-acetyl derivative. In cattle, for instance, the N-glycolyl derivative accounts for 65% of the total plasma sialic acid, while in the hog, 15%, and in the horse, 16% of the total is in this form (25).

γ-Globulin has been reported by a number of investigators (9) to contain glucosamine, galactose, mannose, fucose and sialic acid. Human γ-globulin has also been reported to contain a small amount of uronic acid (equivalent to about 1 residue) (25), but this has not been confirmed by other investigators. As pointed out by Winzler (9), the absolute values for uronic acid content must be considered uncertain in view of the difficulty of determining small amounts of uronic acid in the presence of large amounts of protein.

Rosevear and Smith (28,29) have reported on the isolation of three closely related glycopeptides from a papain hydrolysate of human II-1,2 γ-globulin. The evidence strongly indicates that all three glycopeptides arose from the same structure and that there is a single carbohydrate moiety in the intact glycoprotein which is attached to an aspartyl residue, probably by the β-carboxyl group, which entails an ester or amide bond. The largest glycopeptide was reported to contain 8 glucosamine, 3 galactose, 5 mannose and 2 fucose residues and 1 sialic acid residue. With the possible exception of the glucosamine, the carbohydrate compo-

sition of this glycopeptide accounts for all of the carbohydrate in the intact glycoprotein. The peptide structure of this glycopeptide was given as Glu.Glu.Asp-NH_2.Tyr.Glu.Asp.(Carbohydrate).

Neuberger and coworkers (30-32) have shown hen's egg albumin to contain a single carbohydrate group consisting of about 5 residues of mannose and 3 of glucosamine. In glycopeptides isolated from proteolytic enzyme hydrolysates of egg albumin by Neuberger et al. (31) and by Cunningham et al. (33) and Jevons (34), the carbohydrate group was shown to be linked to an aspartyl residue, as in the case of human II-1,2 γ-globulin.

In studying the chemical composition of α-amylase from A. oryzae, Akabori et al. (35,36) found this enzyme to contain 8 residues of mannose, 1 residue of xylose and 2 residues of hexosamine. A glycopeptide isolated from this enzyme following proteolysis contained all of the mannose and xylose of the intact protein, but no hexosamine. The amino acid sequence of this glycopeptide was suggested to be Ser.Glu.Asp.Gly.(Ala,Thr), and evidence indicates the carbohydrate moiety is linked to the peptide through the hydroxyl group of the seryl residue. The α-amylase from this organism is the only one known to contain carbohydrate (37).

Evidence has been presented which indicates that rabbit γ-globulin, in contrast to human II-1,2 γ-globulin and egg albumin, contains more than one carbohydrate moiety. Porter (38) has isolated three polypeptide fragments with molecular weights of about 50,000 (I), 53,000 (II), and 80,000 (III) from papain hydrolysates of native rabbit γ-globulin and antibodies. Fragments I and III were found to contain both hexose and glucosamine, most of which was in fragment III. Other carbohydrate components were not reported. Fragment II contained little or no carbohydrate (as hexose and glucosamine).

Because the sum of the molecular weights (183,000) of these fragments is in good agreement with the molecular weight obtained for the intact γ-globulin (188,000) and since they were isolated in about 90% yield, it was concluded that these three fragments arose from three different parts of the γ-globulin molecule and, thus, that there is more than one carbohydrate moiety in rabbit γ-globulin. It is interesting that both of the smaller fragments (I and II), when isolated from antibodies, retained the power to combine with antigen but not precipitate it. The largest fragment (III) possessed little or no antibody activity but possessed most of the antigenic specificity of rabbit γ-globulin. Fragments I and II were unable to precipitate or inhibit antibodies to rabbit γ-globulin.

In the experimental work presented in this thesis, glycopeptides isolated from papain hydrolysates of human II-3 γ-globulin, rabbit γ-globulin and antipneumococcal antibody, and immune lactoglobulin from bovine colostrum were studied. Some properties of rabbit γ-globulin have been discussed above. The human γ-globulin and bovine immune lactoglobulin are discussed below.

The II-3 and II-1,2 fractions of human γ-globulin, isolated by the fractional precipitation method of Oncley *et al* (17), have been shown by Smith and coworkers (2) to have identical hexosamine and total hexose contents and very similar amino acid compositions. As previously mentioned, the carbohydrate component of the latter fraction has been examined in greater detail by Rosevear and Smith (29). The II-3 and II-1,2 fractions each arise from γ_2-globulin and possess very similar immunochemical properties. It has been shown that antibodies to a number of antigens were about equally distributed between these two fractions and they appear to

be antigenically indistinguishable (2). These fractions have, however, been shown to contain more than one molecular weight species (2) and differ somewhat in their amino-terminal amino acids, as previously mentioned. These fractions also have different isoelectric points and differ in the extent to which they are soluble in water, fraction II-1,2 being largely a pseudoglobulin and the II-3 fraction largely a euglobulin (2,14).

Throughout fetal development, neither mammals nor birds have the ability to produce antibodies, and it is not until some time after birth that this ability is acquired (39). In man and certain other mammals, including the rhesus monkey, guinea pig and rabbit, the unborn fetus receives passive immunization by the transfer of antibodies from the mother to the fetus. The new-born retains the maternal antibodies until after it begins synthesizing its own antibodies (40). In the ruminants, horse and pig, on the other hand, transfer of antibodies does not occur before birth, and the sera of the new-born of these species contain little or no γ-globulin. In these species the offspring receive protection from infectious diseases through antibodies transmitted by way of the mother's colostrum. In still other species, including dogs, mice and rats, transmission of antibodies to the young occurs both before and after birth (39).

Smith (40) has isolated from the colostrum of a hyperimmunized cow an electrophoretically homogeneous globulin fraction, referred to as "immune lactoglobulin," which accounted for all of the antibody activity of the colostrum. Some physical and chemical properties of the immune lactoglobulin and of pseudoglobulin and euglobulin fractions obtained from it have been compared with γ_2- and T-globulin fractions isolated from the plasma of hyperimmunized cattle (2,40). Both the γ_2-and T-globulin

fractions contained antibody activity. By its electrophoretic mobility and isoelectric point, the immune lactoglobulin has been identified more closely with the T-globulin fraction of plasma than with the main γ-globulin fraction. The proteins of all three fractions were of approximately the same size, and indications were that the molecular weights fell in the range of 160,000 to 190,000 (40). The amino acid compositions of all of these fractions were found to be very similar. Results of hexose and hexosamine analyses indicate the carbohydrate content of the immune lactoglobulin fractions may be somewhat greater than either the γ_2- or T-globulin fractions, particularly with respect to the hexose content (2).

In undertaking studies of these immuno-proteins, it was hoped that information could be gained which would not only disclose in more detail the nature of the carbohydrate component of these glycoproteins but which might also be applicable to the study of other protein-carbohydrate complexes.

II. MATERIALS

In general, the chemicals used in this work were analytical reagent grade. Distilled or deionized-distilled water was used throughout.

Acid glycoprotein, from human plasma (41,42), given to Dr. E. L. Smith by Dr. Weimer and Dr. Winzler, Lot P.

Amino Acids. For calibration of the automatic amino acid analyzer, a Spinco amino acid calibration mixture (No. 120-220, Spinco Division, Beckman Instruments, Inc.) was used. Other amino acids used were commercially available preparations which were recrystallized two to three times and found to be chromatographically pure.

Bovine Immune Lactoglobulin. This preparation was isolated from the colostrum of a hyperimmunized cow by Dr. E. L. Smith (40). The electrophoretic and sedimentation properties and the hexose, hexosamine and partial amino acid composition of this preparation (Fraction B) have been reported (2,40).

Carboxypeptidase, Worthington Biochemical Laboratories, three times recrystallized (C_1 of about 9), treated as described in text before use.

Chymotrypsin, Worthington Biochemical Laboratories, crystalline, salt-free.

Fucose, L(-), Nutritional Biochemical Corp.

Galactose, Merck 3655 (24846), MP 167-168°.

Glucosamine hydrochloride, D(+), Pfanstiehl Laboratories, Inc., 5105, specific rotation: +72.5°. (See Section IV for tests for purity.)

Human γ-globulin, fraction II-3, obtained by Dr. E. L. Smith from E. R. Squibb and Sons, batch 341. The electrophoretic, sedimentation and immunochemical properties and the amino acid, hexose and hexosamine compositions of this preparation have been reported (20,43,44).

Hydrochloric acid, constant boiling, approximately 6 N. All 6 N HCl used for hydrolysis was prepared by distilling a 20% solution in a glass distillation apparatus three times.

Leucine aminopeptidase, prepared from swine kidney by the method of Hill et al. (45). The C_1 of the preparations ranged from 20 to 35.

Orcinol, Eastman 2112, recrystallized once from hot benzene (about 25 g per 700 ml).

Papain, prepared from papaya latex (Wallerstein) by the method of Kimmel and Smith (46). The C_1 of the preparations ranged from 1.0 to 1.5.

Phenylisothiocyanate, Eastman 1484, redistilled, BP 104° at 18 mm.

Prolidase, prepared from swine kidney by the method of Davis and Smith (47). $C_1 = 2$.

Rabbit γ-globulin. Two different preparations of rabbit γ-globulin obtained from E. R. Squibb and Sons were used in this work; both were prepared from rabbits made hyperimmune to Type VII Pneumococcus. The electrophoretic properties, specific antibody content and amino acid composition of one of these preparations have been reported previously (19,21). The electrophoretic properties of the second preparation are reported in Section VI-A-3

Thiobarbituric acid, Eastman 660, recrystallized once from hot water.

III. ANALYTICAL METHODS

A. Amino Acid Analysis

1. Qualitative Analysis. The identification of amino acids was established by paper chromatography in propanol-pyrophosphate buffer (70:30, v/v) (48) and by paper electrophoresis-chromatography. The amino acids were detected by use of the ninhydrin spray reagent of Levy and Chung (49). The presence of hexosamines as well as amino acids can be detected by these procedures.

For chromatography in the propanol-pyrophosphate solvent, Whatman No. 2 paper which had been dipped in the pyrophosphate buffer (0.05 M sodium pyrophosphate, pH 7.2-7.3, 0.1 M NaCl) and dried was used. The chromatograms were developed in a descending system for 17 to 20 hours. The lower edge of the paper was serrated to allow the solvent to drip off.

Paper electrophoresis-chromatography was performed as described by Ingram (50). Whatman No. 3mm paper was used, and the electrophoresis was performed in pyridine-acetate buffer, pH 6.4, at about 14 volts/cm for 3 hours or 40 volts/cm for 45 minutes. The pyridine-acetate buffer used for the lower voltage electrophoresis contained 100 volumes of pyridine, 3.5 volumes of glacial acetic acid and 900 volumes of water; for the higher voltage, this buffer was diluted with an equal volume of water. Electrophoresis at the higher voltage was carried out between water cooled lucite cooling jackets to prevent excessive heating. The electrophoretograms were dried and chromatographed by descending chromato-

graphy in n-butanol-acetic acid-water (200:30:75, v/v) (51) for 17 to 20 hours in a direction at right angles to the direction of electrophoresis. The bottom edge of the paper was serrated to allow the solvent to drip off.

2. Quantitative Analysis. Quantitative estimation of amino acids was achieved by the ion-exchange chromatography methods of Moore, Spackman and Stein (52), either by use of a fraction collector or an automatic recording apparatus (53). Amino sugars, as well as amino acids, can be quantitated by these procedures (52,54).

When the method employing a fraction collector was used, the amino acids and hexosamine emerging from the resin column were quantitated by a photometric ninhydrin method using the ninhydrin color reagent of Moore and Stein (55). The net color yield of each component was converted to leucine equivalents by multiplying the net color yield by the leucine color factor (0.468 μmole/absorbancy unit) determined in this laboratory. The quantity of each component was calculated by dividing the leucine equivalent by its color yield relative to leucine, as established by Moore and Stein (55). For samples containing hexosamine, elution of the long column with pH 4.25 sodium citrate buffer was begun after the column had been eluted with 150 to 160 ml of the pH 3.25 citrate buffer. Under these conditions, the hexosamine emerged after tyrosine and phenylalanine. A detailed description of the apparatus used in this laboratory and the mode of operation has been reported by Kimmel and Smith (56).

The automatic recording apparatus employed in this laboratory is a Spinco Automatic Amino Acid Analyzer. With this instrument, the best separation of hexosamine from amino acids on a long column was achieved by eluting the column with 280 ml of pH 3.25 citrate buffer before

changing to the pH 4.25 citrate buffer. Under these conditions, glucosamine emerges between leucine and tyrosine. The precision of this method is reported to be 100 ± 3% for quantities ranging from 0.25 to 2 micromoles of amino acid (53).

B. Carbohydrate Analysis

1. Qualitative Analysis. Paper chromatography was employed for the identification of carbohydrates. Two solvent systems were used in this work: the n-butanol-pyridine-water (6:4:3, v/v) solvent of Jeanes et al. (57) and sec-butanol-acetone-acetic acid-water (3.0:3.0:1.5:2.5, v/v), developed by Zilliken et al. (58). In addition, hexosamines were detected in conjunction with amino acid analysis by paper chromatography and paper electrophoresis-chromatography, as described in Section III-A-1.

Whatman No. 1 paper was used for chromatography in both of the above solvents, and the chromatograms were developed in descending systems. The lower edge of the paper was serrated to allow the solvent to drip off. When the n-butanol-pyridine-water solvent was employed, the multiple development technique (57) was used extensively to improve the resolution.

The following reagents were used for the detection of sugars on paper chromatograms:

1) p-Anisidine phthalate. (1.2 g p-anisidine, 1.7 g phthalic acid and 10 mg stannous chloride dihydrate dissolved in 100 ml of water-saturated n-butanol) This is the reagent of Hough (59) as modified by Rosevear (60). This reagent is known to give a positive test with aldopentoses, aldohexuronic acids, aldohexoses, 6-deoxyaldohexoses (methylpentoses) and ketoses (60). It does not give a positive test with sialic acids and gives only a weak test with hexosamines.

2) Basic silver. ((a) 2 g $AgNO_3$ dissolved in a minimal amount of water, in 100 ml of methanol. (b) 2 g NaOH dissolved in a minimal amount of water, in 100 ml of methanol) The chromatogram was sprayed with (a) and allowed to dry at room temperature, or it may be dried with the aid of a hair dryer, and then sprayed with (b) and allowed to dry. Darkening of the background obliterates the spots a few minutes after the chromatogram is dry. When desired, this can be prevented by dipping the dry chromatogram into a solution of sodium hydrosulfite acidified with acetic acid, rinsing in water and drying. This reagent, which was developed from the reagent of Trevelyan et al. (61) by Rosevear (60), gives positive tests for sugars and other polyhydroxy compounds. In the case of non-reducing sugars, color development is enhanced by heating.

3) Orcinol-TCA. (0.5 g orcinol and 15 g trichloroacetic acid dissolved in 100 ml of water-saturated n-butanol) The chromatogram was sprayed with the reagent, dried with the aid of a hair dryer and placed in a 105° oven for 15 minutes. Sialic acids give a purple color. This reagent was developed by Klevstrand and Nordal (62) for the detection of ketoheptoses, and it was believed to be specific for these sugars. Blix and co-workers (63), however, found it could be used for the detection of sialic acids.

4) Thiobarbituric acid reagent (64). ((a) 0.05 M sodium periodate in 0.05 N H_2SO_4. (b) ethylene glycol-acetone-concentrated H_2SO_4 (50:50:0.3, v/v). (c) 6% aqueous solution of 2-thiobarbiturate (or an equivalent amount of 2-thiobarbituric acid plus an amount of NaOH equivalent to the amount of thiobarbituric acid used)) The chromatogram was first sprayed with (a) and then, after 15 minutes, with (b). After 10 minutes the chromatogram was sprayed with (c) and heated in a 105° oven for about 10 minutes. Sialic

acids give a red color. This reagent is much more sensitive than the orcinol-TCA reagent, above.

2. Estimation of the Distribution of Hexoses and Fucose. The relative amounts of hexoses and fucose in acid hydrolysates of glycoproteins and glycopeptides were estimated as follows: The hydrolysates were chromatographed on Whatman No. 1 paper (22 1/2 inches in length) in n-butanol-pyridine-water (6:4:3, v/v) as described in Section III-B-1. Use was made of the multiple development technique (24 hours x 2) to obtain the desired degree of resolution. The dry chromatograms were sprayed with p-anisidine phthalate reagent (Section III-B-1). When the colors had been developed, the chromatograms were cut lengthwise into strips 3 cm wide, and the relative intensities of the spots were measured on a Spinco Model R Analytrol, using Cam C (logarithmic) and Wratten No. 45 filters (Kodak). The relative amounts of fucose and hexoses could then be estimated from a knowledge of the relative color yields of these sugars, which were determined by chromatographing known amounts of these sugars and measuring the relative intensities of the spots in the manner described above. The most reproducible results, however, were obtained by simulating the Analytrol pattern of the unknown with known quantities of the component sugars so that the areas under the peaks corresponding to a given component were approximately equal for the unknown and known quantities of sugars.

3. Quantitative Determinations.

a. Photometric Hexose Determination. For this work, the method employed by Winzler (65), as modified by Rosevear and Smith (29), for the determination of protein-bound hexose was employed. These methods are adaptations of the Vasseur modification (66) of the Tillmans-Philippi

orcinol reaction.

Reagents

1) Standard hexose. (2.00 μmoles of hexose (1:1 galactose-mannose) per ml in 10% isopropyl alcohol). 0.1802 g galactose, 0.1802 g mannose, 100 ml isopropyl alcohol, and water to 1 liter.

2) Orcinol-H_2SO_4. (a) 1.6% orcinol (w/v) in 1 N H_2SO_4, 100 ml; and (b) H_2SO_4-water (3:2, v/v), 900 ml. This reagent was kept at 5° and prepared in quantities which would be used up in about two weeks; beyond that time there was a significant increase in the blank. Solutions (a) and (b) are stable indefinitely at room temperature.

Procedure

Samples and standards were placed in 18 mm x 150 mm test tubes and diluted to 1.0 ml with water and isopropyl alcohol so as to give an isopropyl alcohol concentration of 10%. To each tube was added 10 ml of orcinol-H_2SO_4 reagent. The contents of the tubes were mixed thoroughly, and the tubes were placed in an 80° water bath for exactly 15 minutes. The tubes were cooled in cold water, and the color intensities were measured against water in a Coleman Junior Spectrophotometer at a wavelength of 540 mμ. A blank consisting of 1.0 ml of 10% isopropyl alcohol and 10 ml of the color reagent was run with each determination, and the color intensities of the samples and standards were corrected for the blank, the absorbancy of which was usually about 0.002 to 0.003. In addition, when hexose determinations were run on glycopeproteins, the absorbancy of the developed color was corrected for the brown color produced by the action of sulfuric acid on proteins, as suggested by Winzler (65), in the following way: Duplicate samples were run as described above. With these were run a second pair of samples to which 10 ml of solution containing the ingredients of the color

reagent minus the orcinol was added in place of thecolor reagent. The absorbancies of these solutions were subtracted from that of the samples developed with the color reagent. In the case of hexose determinations on glycopeptides, such a correction was found to be unnecessary since the non-specific coloring was negligible. The 1:1 galactose-mannose equivalents of the samples were estimated from a standard curve (absorbancy versus hexose concentration). Such curves were linear up to about 2 μmoles of the standard hexose. Rosevear and Smith (29) report that the isopropyl alcohol improves the linearity of the curve. The net absorbancy of 1.0 μmole of standard hexose was about 0.43.

With the orcinol reagent, the color yield varies from one type of hexose to another, and it is for this reason that Winzler has selected a 1:1 galactose-mannose standard to be used in the estimation of protein-bound hexose in serum proteins, since evidence indicates that serum glycoproteins contain only these two types of hexose (65). However, a knowledge of the fucose content, determined as described in Section III-B-3-c, the ratio of hexoses and fucose (Section III-B-2), and the color yields of the individual components relative to the galactose-mannose standard can be used to obtain a more accurate value for total hexose content. The relative color yields of fucose and some aldohexoses are tabulated in Table 1. The total hexose value, obtained as 1:1 galactose-mannose (G-M) equivalents, was corrected according to the following equation, where m:n is the ratio of galactose to mannose:

$$\mu\text{moles of hexose} = \frac{(\mu\text{moles of 1:1 G-M}) - (0.62)(\mu\text{moles of Fucose})}{(1.11m + 0.88n)/(m + n)}$$

TABLE 1

ORCINOL COLOR YIELDS RELATIVE TO 1:1 GALACTOSE-MANNOSE

Carbohydrate	Color Yields[a]
Galactose	1.11
Mannose	0.88
Glucose	0.37
Fucose	0.62
Galactose-Mannose, 3:5	0.98

[a] From the data of Rosevear and Smith (29)

Hexosamine and sialic acids have been reported not to interfere with this determination (63,65,66). Winzler (65) has reported that fucose also does not interfere; however, as indicated in Table 1, Rosevear and Smith have found that a color is produced with fucose. This finding has been confirmed in this work.

b. Hexosamine Determination. Two methods were employed for the quantitative estimation of hexosamine. One of these methods, in which hexosamine was determined in conjunction with the quantitative analysis of amino acids by ion-exchange chromatography, is described in Section III-A-2. The second method employed is the photometric method introduced by Levvy and McAllan (67). A method which has been used extensively for the determination of hexosamine is the photometric procedure of Elson and Morgan (68). In this procedure and its numerous modifications (54,65,69-72) hexosamine is reacted with acetylacetone to form a chromagen, which, in turn, forms a chromophore with a p-dimethylaminobenzaldehyde reagent. The Levvy and McAllan method employs acetic anhydride in place of acetylacetone, and

the p-dimethylaminobenzaldehyde reagent of Reissig et al. (73) is used to estimate the N-acetyl-hexosamine formed. The acetylation reaction has been reported to be more specific than the reaction involving acetylacetone in that other sugars and amino acids do not interfere (74). In addition, with the use of the color reagent of Reissig et al., there is much less interference by chromagens formed by the interaction of sugars and amino acids than there is with color reagents used previously (73). The method has the advantage of greater sensitivity, convenience and reproducibility, and the degree of color development is not sensitive to small amounts of acid or base in the sample, as it is in older methods (67).

Reagents

1) HCl, 4.0 N.

2) Acetic Anhydride, 1.5% (v/v) solution in acetone (prepared daily).

3) p-Dimethylaminobenzaldehyde, 10 g (Eastman) dissolved in 100 ml of glacial acetic acid containing 12.5% (v/v) 10 N HCl. This solution was stored at 5° and was diluted with 9 volumes of glacial acetic acid just before use.

4) Borate buffer, 0.7 M with respect to $B_4O_7^=$, pH 9.2. To a 100 ml volumetric flask containing approximately 80 ml of water and 5 to 6 g of KOH was added 17.32 g of boric acid, which was dissolved with the aid of warming. The solution was then cooled, adjusted to pH 9.2 with additional KOH and diluted to 100 ml with water.

5) Standard hexosamine (1.00 μmole/ml), 0.1078 g of glucosamine hydrochloride and water to 500 ml.

Procedure

All steps of the assay procedure, including acid hydrolysis of the

sample, where necessary, were performed in a single tube.

Acid hydrolysis. To 1 ml of sample in a glass-stoppered tube, 15 mm x 115 mm, was added 1 ml of 4.0 N HCl. The tube was stoppered and placed in a 97° to 100° wax bath for 12 hours. Stoppers were held in place with wire clamps. To reduce loss by evaporation, the tubes were placed in the bath so that approximately two-thirds of the tube length was above the surface of the wax. Where loss by evaporation was appreciable, low glucosamine values were obtained with standard glucosamine samples. At the end of the 12 hour hydrolysis period the tubes were removed from the bath and the contents were immediately frozen and freeze-dried.

Assay procedure. Samples and standards were diluted to 0.6 ml with water. To an additional tube was added 0.6 ml of water to be run as a blank. To each tube was added 0.1 ml of acetic anhydride reagent followed by 0.5 ml of borate buffer. The solutions were then swirled to insure washing down of the tube walls, and the tubes were stoppered and the stoppers clamped into place, as before. The tubes were placed in a vigorously boiling water bath for exactly 3 minutes and then cooled in cold tap water. To each tube was added 6 ml of freshly prepared p-dimethylaminobenzaldehyde reagent. The tubes were stoppered, their contents mixed by inversion, and placed in a 40° water bath for 20 minutes. The tubes were cooled to room temperature in a water bath, and the intensity of the developed color was read almost immediately against the blank with a Zeiss PMQII Spectrophotometer at a wavelength of 545 mμ. The quantity of glucosamine in the unknown was estimated from a standard curve. The absorbancy is proportional to the concentration up to approximately 0.3 μmole of glucosamine per sample. Standard glucosamine subjected to a simulated acid

hydrolysis in the presence of the amino acids phenylalanine, aspartic acid and glutamic acid under the hydrolysis conditions described above and assayed according to the above procedure was recovered from duplicate samples to the extent of 99 ± 1% (Section IV).

c. Photometric Fucose Determination. For the quantitative estimation of fucose, the photometric method of Dische and Shettles (75), as applied to the determination of fucose in serum proteins by Winzler (65), was used. The 3 minute rather than the 10 minute heating period was used. Absorbancies at 396 and 430 mμ were read on a Zeiss PMQII Spectrophotometer or a Beckman DU Spectrophotometer. Reagent blanks were run with each determination, and the net absorbancy was calculated according to the equation:

$$\text{Net } A = [(S'_{396} - B'_{396}) - (S'_{430} - B'_{430})] - [(S_{396} - B_{396}) - (S_{430} - B_{430})]$$

where,

S' = Absorbancy of sample with cysteine

S = Absorbancy of sample without cysteine

B' = Absorbancy of blank with cysteine

B = Absorbancy of blank without cysteine.

The quantity of fucose in the unknowns was estimated from a standard curve constructed with the use of an external standard of L-fucose.

d. Photometric Sialic Acid Determination. The quantitative estimation of sialic acids was carried out by one of two methods. In the earlier work, the direct Ehrlich method of Werner and Odin (76) was used. The acid glycoprotein of Weimer, Mehl and Winzler (41), which was found to contain 10.1% sialic acid by this method (76), was used as a standard for the assay.

The thiobarbituric acid method for the determination of sialic acid has been used in this work since it was introduced by Warren (77) in 1959. This procedure has the advantage of having greater specificity and sensi-

tivity than the methods previously available. An additional important advantage this procedure has over earlier methods is that the color yields are the same for the different types of sialic acids since the chromagens formed are the same. Of the substances known to interfere with the determination of sialic acids by this procedure, only L-fucose is present in the materials analyzed in this work. Among the substances found not to interfere with this determination are galactosamine, glucosamine, N-acetylglucosamine, glucose, galactose, mannose, aspartic acid and glutamic acid. Warren has reported that when 0.04 μmole of N-acetylneuraminic acid was assayed in the presence of 1 μmole of fucose, the absorbancy was decreased 35% below that of a sample containing no fucose (77). The absolute amount of fucose and the molar ratios of fucose and sialic acid in the samples analyzed in this work were many times smaller than this, and, therefore, the interference due to fucose in these samples would not be expected to be very great. To estimate the extent of inhibition due to fucose in the materials analyzed in this study, aliquots of a glycopeptide preparation from rabbit γ-globulin, the fucose content of which was known, were assayed in the presence of graded amounts of authentic fucose (Section VI-A-3-(iii)). The per cent transmission of the developed colors was found to be a straight line function of the total fucose concentration. The per cent transmission obtained by extrapolating the curve to zero fucose concentration was used to calculate the sialic acid content of the sample. This corrected value was only 6% higher than the uncorrected value.

C. Nitrogen Determination

The nitrogen content of proteins was determined by the titrimetric micro-Kjeldahl technique described by Van Slyke *et al.* (78).

IV. STUDIES ON THE DESTRUCTION OF GLUCOSAMINE IN ACID SOLUTION

A. Introduction

The most widely used methods for the quantitative analysis of hexosamine in biological materials involve hydrolysis of the materials with hydrochloric acid and analysis of the hydrolysate by colorimetric procedures. Hexosamine analysis by these procedures presents a two-fold problem. First, there is the problem of the presence in these materials of substances which interfere with the colorimetric determination of hexosamine. The relative merits in this respect of some commonly used procedures have been discussed previously (Section III-B-3-b). The problem of interfering substances has been circumvented by some workers by employing more time-consuming procedures in which hexosamine is isolated from the acid hydrolysate prior to colorimetric analysis. This procedure, however, does not eliminate the second problem, that of the quantitative release of hexosamine by acid hydrolysis without destruction of the hexosamine. Since the only hexosamine found in the glycoproteins and glycopeptides studied in the present work is glucosamine, only this hexosamine will be considered in the discussion to follow.

Boas (71) found by heating authentic glucosamine hydrochloride for 15 hours at 100° in 2, 3, 4, 5 and 6 normal hydrochloric acid that glucosamine was partially destroyed under these conditions in acid concentrations greater than 2 normal. The extent of destruction increased with increasing acid concentration up to about 20% in 6 N hydrochloric acid.

Thus, with respect to destruction, lower concentrations of acid are preferable. Generally, glycosidic bonds would be readily cleaved under these milder conditions. In the case of hexosamines, however, the situation is complicated by the presence of the amino group in two ways. First, the possibility exists that hexosamine may occur in mucoproteins linked to amino acids by an amide bond, which would be more resistant to acid hydrolysis than glycosidic linkages. The second and probably the most important complication is that the proximity of a positively charged amino group to a glycosidic linkage, especially those involving the 1 or 3 carbon of a 2-deoxyaminohexose, greatly increases the resistance of such glycosidic bonds to acid hydrolysis (32). Glucosamine nearly always occurs in biological materials as the N-acetyl derivative (79), and there is evidence that it occurs in this form in the glycoproteins studied in this work. Thus, in these substances the amino group of glucosamine probably does not carry a positive charge. However, as pointed out by Neuberger et al. (32), the hydrolysis of N-acetylglucosaminides may procede by two different pathways. Hydrolysis of the glycosidic bond may occur first, followed by hydrolysis of the acetamido bond, or the cleavage of these two bonds may occur in the reverse order. In the latter case, the glycosidic bond becomes more resistant to hydrolysis due to the presence of the free amino group. In studies with methyl N-acetylglucosaminide and methyl glucosaminide, Neuberger and coworkers (32) found the rate of cleavage of the glycosidic linkage of α-methyl N-acetylglucosaminide in 1 N hydrochloric acid at 100° is of the order of 250 times greater than the rate of cleavage of this bond in α-methyl glucosaminide. The preferred pathway, that in which the glycosidic bond is broken first, seems to be favored over the alternate pathway by an

increase in the hydrolysis temperature, as predicted from the heats of activation of the glycosidic and acetamido bonds of α-methyl N-acetylglucosaminide. For example, in 1 N hydrochloric acid the rate of hydrolysis of the glycosidic bond of this compound was found to be 3.4 times greater than the rate of hydrolysis of the acetamido bond at 80°, while at 61.25° the rate was only 1.6 times greater. Also, the rate of hydrolysis of methyl glucosaminide at 100° was nearly five times greater in 2.5 N hydrochloric acid than in 1 N hydrochloric acid (32).

Thus, to obtain maximum recovery of hexosamine, it appears a compromise must be made between conditions which will insure complete hydrolysis and those which minimize hexosamine destruction. As demonstrated by Boas (71), the conditions for maximum recovery of glucosamine from various tissues vary with the tissue, and, thus, it would be desirable to determine these conditions for each tissue or substance studied. Boas obtained the maximum yield of glucosamine from blood plasma after hydrolysis in 1 to 2 N hydrochloric acid for 15 hours at 100°. In extensive studies on the recovery of glucosamine from hydrolysates of hen's egg albumin, Neuberger et al. (32) obtained a maximum value for the glucosamine content (1.2%) of this glycoprotein by hydrolysis for 3 or 6 hours at 100° in 4 and 5.7 N hydrochloric acid; the recovery after hydrolysis at 100° in 2 N hydrochloric acid for 3 hours was only about 80% of this maximum value. Sorenson (80), however, found 1.2% glucosamine in egg albumin after hydrolysis in 1 N hydrochloric acid for 12 hours at 100° and 1.4% with 5 N hydrochloric acid for 3 or 6 hours at the same temperature.

In the present studies, the glucosamine content of glycopeptides has been determined by the photometric procedure of Levvy and McAllan (Section

III-B-3-b) and by the ion-exchange chromatography method of Moore, Spackman and Stein (Section III-A-2) in conjunction with amino acid analyses. For analysis by the former method, the samples were hydrolyzed in 2 N hydrochloric acid for 12 hours at 97° to 100°; in the latter method, the samples were hydrolyzed in 6 N hydrochloric acid for 20 to 21 hours at 110°. The large discrepancies in the values obtained by the two methods indicated that glucosamine destruction under the latter conditions might be considerably greater than indicated by the work of Boas and prompted a study of glucosamine destruction under the conditions employed in these assay procedures.

B. Experimental

Although it would be desirable to determine the optimum conditions for acid hydrolysis of the glycopeptides and the extent of destruction under these conditions by varying the conditions of hydrolysis, the lack of sufficient quantities of the glycopeptides did not permit such studies. To obtain an indication of the extent of glucosamine destruction under the conditions employed in the assay of the glycopeptides, solutions of authentic glucosamine were treated with hydrochloric acid in the presence of the amino acids and neutral sugars found in the acid hydrolysates of the glycopeptides under the conditions described for the assay of glucosamine in these glycopeptides. The molar ratios of these authentic compounds were approximately the same as in the glycopeptides studied.

Standard Solutions. Standard solutions of glucosamine hydrochloride, aspartic acid, glutamic acid, phenylalanine, tyrosine, galactose, mannose, and fucose were prepared. Paper chromatography of portions of the standard glucosamine in n-butanol-pyridine-water (6:4:3) indicated the preparation was pure; only a single spot could be detected with p-anisidine phthalate,

basic silver and ninhydrin reagents (Section III-B-1). The glucosamine hydrochloride was dissolved in 0.2 N hydrochloric acid and prepared fresh weekly. The standard was stored at 5°.

Experiment 1. From the standard solutions was prepared a solution containing glucosamine, 1.0; Asp, 0.4; Glu, 0.2; Tyr, 0.2, galactose, 0.6; mannose, 1.0; and fucose, 0.4 μmole/ml. This solution simulates the analytical composition of glycopeptide 3 from human II-3 γ-globulin (Table 5), except the glucosamine concentration in the solution is equivalent to 5 rather than 3 residues, as found in this glycopeptide before correction for destruction. One ml aliquots of this solution were concentrated to dryness and the residues were treated as described below and analyzed by the method of Moore, Spackman and Stein on an automatic amino acid analyzer. (a) Two untreated samples were analyzed directly. (b) A second sample was heated in 6 N hydrochloric acid at 110° in the presence of 0.12 g of sodium sulfite (equivalent to 0.1 g of sodium bisulfite) for 21 hours, exactly as described for glycopeptide 3 from human II-3 γ-globulin (Section V-A-3-a). (c) A third sample was treated as described under (b) except sodium sulfite was omitted.

Experiment 2. A second solution was made up to simulate the glucosamine and amino acid composition of glycopeptide 2 from rabbit γ-globulin (Table 9). An untreated 1 ml aliquot (a) of the solution, containing glucosamine, 0.80; Asp, 0.26; Glu, 0.53; and Phe, 0.26 μmole, was analyzed on the automatic amino acid analyzer. A second 1 ml aliquot (b) was treated with 6 N hydrochloric acid at 110° for 20 hours and analyzed in the same manner. The conditions described for the hydrolysis and analysis of the glycopeptides isolated from rabbit γ-globulin were duplicated as nearly as possible (Section

VI-A-3-b-(ii)).

Experiment 3. Duplicate aliquots of the glucosamine-amino acid solution described in Experiment 2, containing 0.2 μmole of glucosamine, were analyzed for glucosamine by the method of Levvy and McAllan after they had been heated in 2 N hydrochloric acid for 12 hours at 97° to 100°. These are the conditions employed for the hydrolysis of the rabbit glycopeptides prior to glucosamine analysis by this procedure (Section VI-A-3-(iii)).

Experiment 4. Aliquots of the glucosamine stock solution, the theoretical glucosamine content of which was 0.20 μmole, were analyzed for glucosamine by the photometric ninhydrin procedure of Moore and Stein (55) using leucine as a standard. The analysis was performed in triplicate (three aliquots of glucosamine standard and three 0.20 μmole aliquots of leucine standard), and the conditions outlined by these authors were followed exactly except a 20 minute rather than a 15 minute heating period was used to develop the ninhydrin colors. The absorbancies of the glucosamine samples were converted to leucine equivalents and the color yield of glucosamine (1.03) relative to leucine determined by Moore and Stein was used to calculate the glucosamine content of the samples.

The results of these experiments are tabulated in Table 2. The glucosamine values obtained by analysis on the automatic amino acid analyzer are based on a constant calculated from the constant for glucosamine reported by Spackman, Stein and Moore (53). Methionine, isoleucine, leucine, tyrosine and phenylalanine, like glucosamine, emerge from the long resin column after the pH 4.25 buffer front. The average ratio of the constants for these amino acids reported by these authors to the constants determined for these amino acids on the automatic amino acid analyzer used in the present work

is 1.24 ± 0.02. The constant for glucosamine reported by Spackman et al. was divided by this factor to obtain the constant for glucosamine, 21.7, used both in the above destruction studies and in calculating the glucosamine content of the glycopeptides.

TABLE 2

GLUCOSAMINE DESTRUCTION STUDIES

Component[a]	% Recovery					
	Experiment Number					
	1(a)	1(b)	1(c)	2	3	4
Glucosamine	85, 73	56	49	53	99 ± 1	97 ± 3
Aspartic Acid	94, 95	95	97	98	-	-
Glutamic Acid	96, 87	94	100	92	-	-
Tyrosine	100, 94	94	93	-	-	-

[a] Phenylalanine was lost from the determination in Experiment 2.

C. Conclusions

The results of experiment 4, together with the chromatographic tests for purity, indicate that the glucosamine standard used in these studies is highly pure. Destruction of glucosamine in the presence of amino acids (Experiment 2) and in the presence of both amino acids and neutral sugars (Experiments 1(b) and 1(c)) found in the glycopeptides under the conditions employed for analysis by the ion-exchange chromatography method of Moore, Spackman and Stein (Section III-A-2) is about 45 to 50%. The presence of sodium sulfite during the acid treatment of these standard solutions (Experiment 1(b)) had little or no sparing effect on the glucosamine. Experiment

1(a), in which the untreated standards were analyzed directly, indicates that part of the destruction of glucosamine under the conditions of the analysis is due to destruction on the ion-exchange column. It was found that in the case of the lower recovery value for glucosamine (73%) in this experiment the sample remained on the resin column several hours longer than usual; due to a power failure, elution was not begun until 5 or 6 hours after the sample was applied to the column. Ordinarily, glucosamine is eluted from the column at 50° in about 18 hours. As can be seen from Experiments 1(a), 1(b), 1(c) and 2, there was little or no destruction of the amino acids in the presence of glucosamine and the neutral sugars galactose, mannose and fucose; the recovery from the acid treated samples was the same, within the limits of accuracy, as the recovery from the untreated samples. Experiment 3 demonstrates that little or no destruction of glucosamine occurs in the presence of the amino acids found in the rabbit glycopeptides under the hydrolytic conditions employed for glucosamine analysis of these glycopeptides by the method of Levvy and McAllan. It has not been demonstrated, however, that complete hydrolysis is attained under these conditions.

If, as indicated by these experiments, a significant part of the total glucosamine destruction under the conditions employed for analysis on the amino acid analyzer occurred on the resin column of the analyzer, the question then arises as to why more destruction occurred on the column in these studies than occurred in the hands of Spackman, Stein and Moore. The answer to this question is not apparent. The glucosamine constant reported by these investigators (53) is about 4% lower than that reported for leucine, while the color yield of glucosamine, as determined by a direct photometric ninhydrin method, has been reported to be 1.03 times greater than that of

leucine (55). This indicates that some (about 7%) glucosamine destruction occurred on the analyzer resin column in the hands of Spackman, Stein and Moore. However, since the same glucosamine constant was used in these destruction studies as in the analysis of the glycopeptides and the conditions of the analyses were standardized, the total recoveries can be related, regardless of the cause of destruction.

Although destruction factors obtained in this way must be used with caution, the results of these experiments are in line with data published by Neuberger et al. (32) after these studies were concluded. These authors found that after hydrolysis of a glycopeptide from egg albumin in 5.7 N hydrochloric acid at 115° for 36 hours, the glucosamine recovered, as determined by a modification of the ion-exchange chromatography method of Moore and Stein, was only 46% of the amount found after hydrolysis for only 18 hours under the same conditions.

V. HUMAN II-3 γ-GLOBULIN

A. Experimental Methods and Results

1. Carbohydrate Composition. The identification of the component carbohydrates of human II-3 γ-globulin was accomplished by paper chromatography. The samples were hydrolyzed with dilute sulfuric acid and the hydrolysates prepared for chromatography according to the following procedures:

For the detection of sugars other than sialic acid, 5 ml of 0.25 N H_2SO_4 was added to 50 to 100 mg of dry, salt-free sample in a hydrolysis tube. The tube was evacuated and sealed and placed in a 105° oven for 17 to 18 hours, according to the procedure of Gottschalk and Ada (81). The brown hydrolysate was cooled and titrated with a saturated solution of barium hydroxide to pH 7.2 to 7.3, and the excess barium ion was precipitated by bubbling carbon dioxide into the suspension to a pH of about 5. The free sugars were separated from the barium sulfate and barium carbonate precipitates and larger, soluble molecular species, i.e., proteins or large peptides, by dialyzing the suspension against water (3 volumes x 5) at 5° in Visking 23/32 dialysis tubing. A Craig-type dialysis apparatus (82) was used to increase the dialyzing surface. The combined dialysates, which were clear and contained only a small amount of the brown coloration present in the hydrolysate, were concentrated to dryness on a rotary evaporator at 25 to 30°. Further purification

of the hydrolysate was accomplished by one of two means: (a) The residue was extracted with small portions of a 10% solution of trichloroacetic acid in water, the combined extracts were centrifuged to remove insolubles, the trichloroacetic acid was extracted from the supernatant solution with diethyl ether (1 volume x 6) and the aqueous phase was concentrated to a convenient volume for chromatography; or (b) the residue was dissolved in 1 to 2 ml of water and applied to a 0.5 cm x 20 cm column of Dowex 50-X2 in the hydrogen cycle, the sugars, except hexosamine, were eluted from the column with 3 column volumes of deionized-distilled water, and the eluate was concentrated to a convenient volume for chromatography. Hexosamine can be eluted from such a column with 2 N HCl.

Paper chromatography was performed in a n-butanol-pyridine-water (6:4:3) system, and duplicate chromatograms were sprayed, one with p-anisidine phthalate reagent and the other with basic silver reagent, as described in Section III-B-1. The chromatograms of samples prepared in this way were essentially free of streaking in the cases where the p-anisidine phthalate reagent was used. Only a small amount of streaking was noted on chromatograms sprayed with the less specific basic silver reagent.

To test for sialic acids, which will not survive the above hydrolysis conditions, 25 to 50 mg of the dry, salt-free γ-globulin was heated in a glass-stoppered tube with 5 ml of 0.05 N H_2SO_4 at 90°, or with 5 ml of 0.1 N H_2SO_4 at 80°, for 1 hour. These conditions were found by Svennerholm (83) to give the maximum yield of sialic acids from a variety of tissues. The hydrolysates were cooled and treated with barium hydroxide and carbon dioxide and dialyzed against water as described above. The combined dialysates were concentrated to a convenient volume for chromatography. Chromatography was

performed in the n-butanol-pyridine-water solvent and the chromatograms were sprayed with thiobarbituric acid reagent or orcinol-TCA reagent (Section III-B-1).

By comparison with authentic sugars chromatographed with the samples, the component sugars of human II-3 γ-globulin were identified as glucosamine, galactose, mannose, fucose and sialic acid. Pentoses, ketohexoses, hexuronic acids and other aldohexoses would have been detected had they been present.

The hexosamine content of this same preparation of II-3 γ-globulin has been reported by Smith et al. (84) to be 1.23%, as estimated by a modification of the Elson and Morgan method. With the use of glucose as a standard and an orcinol-H_2SO_4 method different from that employed in the present study, these workers found the total hexose content of this preparation to be 2.34%. If it can be assumed that the relative color yields listed in Table 1 apply to both of these orcinol-H_2SO_4 methods, then the total hexose value as 1:1 galactose-mannose equivalents is 0.87%. When this value is corrected for the fucose content and the ratio of galactose to mannose (3:5) found in glycopeptides isolated from this protein preparation (Section V-A-3-b), the value for hexose, as 3:5 galactose-mannose, becomes 0.77%, or 7.58 moles per mole of protein (based on a molecular weight of 160,000 for γ-globulin). This is equivalent to a total of 7 or 8 residues of galactose and mannose per molecule of protein.

2. Preparation of Glycopeptides. To further characterize the carbohydrate component, the γ-globulin was digested with the proteolytic enzyme papain, and the resulting glycopeptides were isolated from the digest by the procedure developed by Rosevear and Smith (29), as outlined below.

Step 1: Digestion with Papain. To 10 g of the salt-free γ-globulin suspended in 50 ml of water was added 0.1 g of recrystallized mercuripapain (C_1=1.0). The mercuripapain was activated by the addition of BAL (2,3-dimercaptopropanol) to a concentration of 0.005 M. The digestion was performed at 60° in a constant temperature water bath. The pH of the digestion mixture was maintained between 6.3 and 6.8 by the intermittent addition of 1 N NaOH. Mercuripapain and BAL were added to the digestion mixture at intervals during the course of the digestion to a total of 0.2 g of the enzyme. The digestion was allowed to continue until there was no increase in the photometric ninhydrin color value; this required 69 hours. At the end of the digestion period, the digestion mixture was centrifuged to remove oxidized BAL and a small amount of other insolubles. The residue was discarded and the clear supernatant solution was saved for step 2.

Papain is highly active at 60°, and digestion at this temperature has the added advantage of reducing the possibility of bacterial growth. Papain is suitable for use in this work since it contains no carbohydrate (85,86) and its broad specificity enables it to digest proteins extensively. Rosevear and Smith have (29) reported that a fraction of human γ-globulin digested with papain as described above was hydrolyzed to the extent of about 50%. The properties of papain have been reviewed by Kimmel and Smith (86,87).

Step 2: Dowex 50 Treatment. The supernatant solution from step 1 was passed through a 2.2 cm x 13 cm column of Dowex 50-X8 resin (20 to 50 mesh) in the hydrogen cycle at 5°. The resin had previously been thoroughly washed with deionized-distilled water. The eluate was allowed to flow from the column at a rapid drop-wise rate and was recycled through the column

two times. The column was then washed with 2 column volumes of cold water, and the pH of the combined eluates was immediately adjusted to 7.0 with 1 N LiOH at 5°. The nature of the papain digestion mixture and the glycopeptides lends itself to the purification of the glycopeptides by this procedure. The electronegative character and relatively large size of the glycopeptides permit most of the glycopeptide material to pass through the resin. The fragments which do not contain carbohydrate are much smaller on the average, and, consequently, this material is more readily bound by the resin.

Step 3: Ethanol Precipitation. The combined resin column eluates were concentrated to a more convenient volume (50 ml) and added with stirring to a volume of absolute ethanol such that the final ethanol concentration was 92.5% (v/v). A saturated solution of LiCl in ethanol (0.2 to 0.5 ml) was added to the cloudy mixture to promote flocculation of the precipitate. The mixture was allowed to stand at 5° until the precipitate had settled to the bottom of the container and the supernatant solution was clear. Additional LiCl solution was added as needed. Most of the supernatant solution was removed by siphoning, and the precipitate was recovered from the remaining supernatant by centrifugation at 5°. The supernatant was discarded and the precipitate was washed with cold absolute ethanol and cold anhydrous ether. The precipitate was dissolved in the smallest possible volume of water and the precipitation process repeated two times. The final precipitate was dried *in vacuo* over P_2O_5. The yield was 435 mg.

Step 4: Zone Electrophoresis. By the use of paper strip electrophoresis it was found that three components which migrated toward the positive pole in pH 8.6 Veronal buffer were the major ninhydrin-positive substances present in the carbohydrate-rich material obtained from step 3.

No positively charged ninhydrin-positive components could be detected. Electrophoresis was performed on Whatman No. 3 mm paper strips, 3 cm x 30 cm, with a Spinco Model R paper electrophoresis apparatus in 0.05 N Veronal buffer, pH 8.6. Approximately 1 mg of sample in 0.01 ml of buffer was applied in a narrow band across the width of the strip with a Spinco sample applicator. The electrophoresis was performed at 5°. Good resolution was obtained in 20 hours with 0.6 ma per strip (constant current) and an initial voltage of 175 volts. The dry strips were sprayed with the ninhydrin reagent of Levy and Chung (88), and the colors were developed by gentle heating with a hair dryer.

The remaining material obtained from step 3 was further purified by starch column electrophoresis. Column electrophoresis techniques have been described by Porath et al. (89,90) and by Flodin and Kupke (91). The electrophoresis apparatus used in this work is similar to that described in reference 89 and has been described in detail by other members of this laboratory (45). Electrophoresis was performed at 5° on a 2.8 cm x 50 cm column in 0.05 N Veronal buffer, pH 8.6. The positive electrode was placed at the bottom of the column since the glycopeptides were found by paper strip electrophoresis to be negatively charged at this pH. The buffer was prepared as follows: To 82.5 g of sodium diethylbarbiturate dissolved in water was added 800 ml of isopropyl alcohol in which had been dissolved, with warming, 16.0 g of diethylbarbituric acid. This solution was diluted to 8.0 liters with water. The isopropyl alcohol, which was added as a preservative, also facilitated the dissolution of the diethylbarbituric acid. Two-hundred mg of sample dissolved in 0.4 ml of buffer was applied to the column and washed into the starch with small portions of

buffer. The electrophoresis was performed for 72 hours at a constant potential of 425 volts; the current was 10 to 11 ma.

Following electrophoresis, the starch column was placed on an automatic fraction collector and eluted with the Veronal buffer at 5°. The column effluent was collected in 0.5 ml fractions, and 0.1 ml of every fifth fraction was assayed for hexose by the orcinol-H_2SO_4 method (Section III-B-3-a). Equal aliquots from the same fractions were assayed for ninhydrin-reactive material by a photometric ninhydrin procedure (55). By plotting the absorbancies against the fraction number, an elution diagram such as that shown in Figure 1 was obtained. The three major components which are both ninhydrin- and orcinol-positive are designated glycopeptides 1, 2 and 3, in order of their emergence from the column (and, thus, in order of decreasing electronegativity). The elution pattern is very similar to that obtained by Rosevear and Smith (29) in the isolation of glycopeptides from human II-1,2 γ-globulin. The center fractions of each of these three glycopeptide peaks were combined as indicated in Figure 1. The glycopeptide fractions from two such starch electrophoresis runs were combined and filtered through a medium sintered glass funnel, and the glycopeptides were precipitated from 92.5% ethanol, washed with cold absolute ethanol and cold anhydrous ether and dried *in vacuo* over P_2O_5 as described in step 3. From 400 mg of material from step 3, the yields of glycopeptides 1, 2 and 3 were 18, 29 and 21 mg, respectively.

3. Characterization of Glycopeptides. For further study, stock solutions of the glycopeptides obtained from step 4 of Section V-A-2 were prepared. The glycopeptides were dissolved in lint-free deionized-distilled

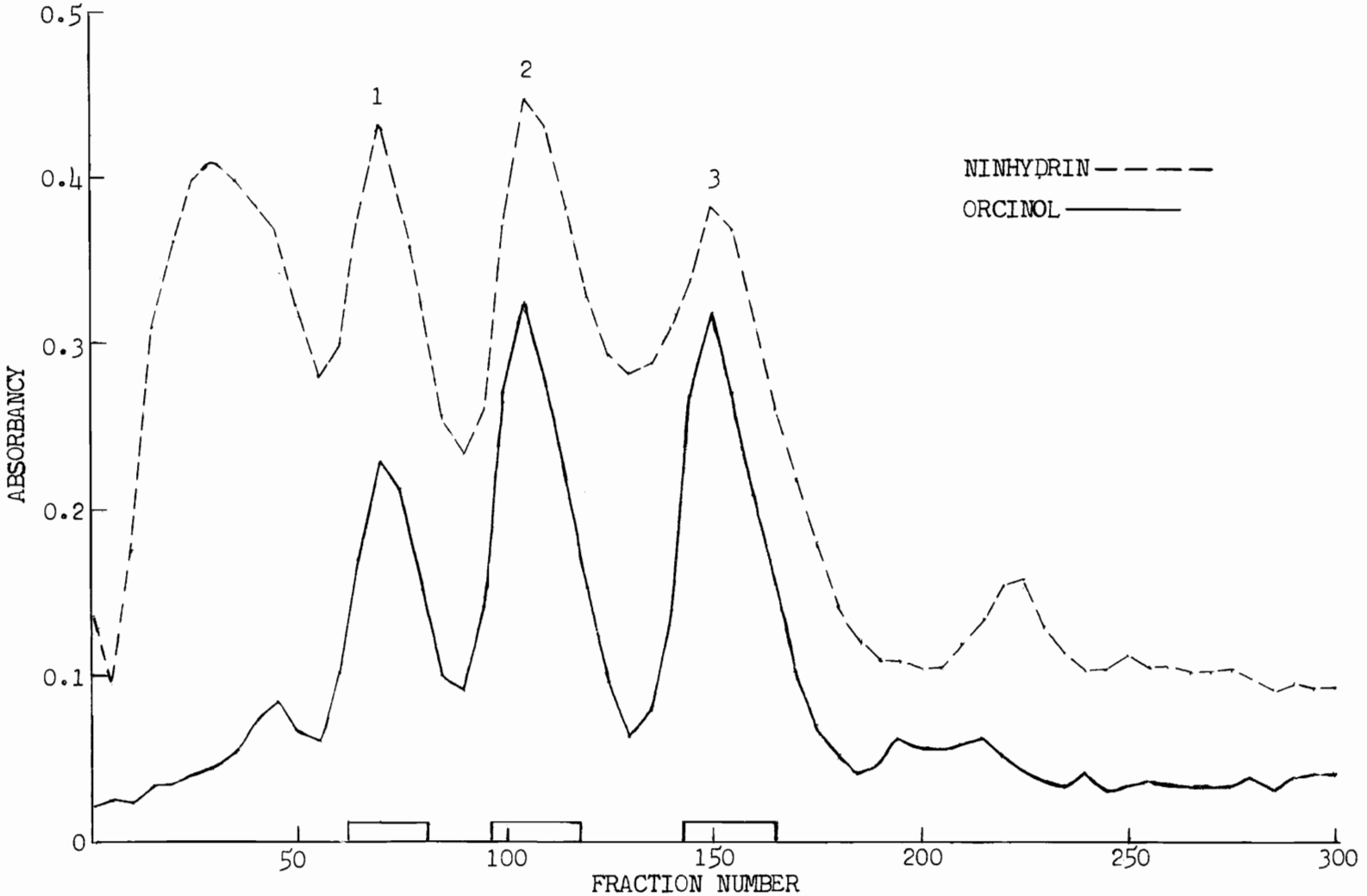

Figure 1. Starch column electrophoresis elution diagram of glycopeptides from human II-3 γ-globulin. Electrophoresis was performed in 0.05 M Veronal buffer, pH 8.6. Effluent fractions contained 0.5 ml each. The block under each peak represents the fractions pooled.

water to give a concentration of approximately 2 mg/ml.

a. Amino Acid Composition. The amino acids present in acid hydrolysates of the three glycopeptides were identified by means of paper electrophoresis-chromatography, as described in Section III-A-1. Aliquots of the stock solutions were hydrolyzed in 6 N HCl at 105° for 20 hours in sealed capillary tubes. The hydrolysates were concentrated to dryness in vacuo over NaOH, and the residues were dissolved in a convenient volume of pyridine-acetate buffer for electrophoresis. The major ninhydrin-positive components of each of the glycopeptides were found to be glucosamine, glutamic acid, aspartic acid and tyrosine; phenylalanine and serine and/or glycine were present in somewhat smaller amounts in each case. (Serine and glycine are not completely resolved in the chromatographic system employed.) Other amino acids were either absent or present in only trace amounts.

Quantitative amino acid analysis was accomplished by means of the ion-exchange chromatography procedures of Moore, Spackman and Stein, described in Section III-A-2. The procedure which employs an automatic fraction collector to collect the resin column effluent was used for the amino acid analysis of glycopeptides 1 and 2; glycopeptide 3 was analyzed on an automatic amino acid analyzer. Since basic amino acids were present in only trace amounts, only a long column analysis was performed in each case. A 2.0 ml aliquot of each stock solution, containing approximately 4 mg of glycopeptide, was placed in a hydrolysis tube and concentrated to dryness on a rotary evaporator. To each of these samples was added 5 ml of 6 N HCl. The acid solutions were frozen in an alcohol-dry ice bath. According to the procedure followed by Rosevear (60), a quantity of sodium bisulfite calculated to produce 1 atmosphere of SO_2 at 105° was added to each tube

(0.1 g of sodium bisulfite in a 20 ml tube), and the tubes were evacuated and sealed while the acid solutions were still frozen. Hydrolysis was performed at 105° for 20 to 22 hours. The hydrolysates were concentrated to dryness on a rotary evaporator and dissolved in 0.2 N sodium citrate buffer, pH 2.2, for application to the ion-exchange column.

The use of SO_2 has been recommended by Pederson and Baker (92) to reduce the destruction of amino acids during acid hydrolysis, and Hodge and Rist (93) have reported that bisulfite reduces the destruction of amino acids in the presence of N-glycosides, the latter of which may be formed by heating reducing sugars in the presence of amines. These workers have proposed that these reactions are involved in the so-called "browning reaction" between amino acids and sugars. However, this sequence of reactions occurs most readily at neutral or alkaline pH. Hoover et al. (94) have reported that when 0.3 g of fibrin was hydrolyzed in 6 N HCl for 48 hours at 100° in the presence of 1.5 g of glucose, the recovery of lysine from the hydrolysate was only 15% below that obtained by hydrolyzing the protein under the same conditions in the absence of glucose.

The results of the amino acid analyses are tabulated in Tables 3, 4 and 5. The values listed are not corrected for destruction. Because of the "browning reaction," the degree of destruction of the amino acids might be expected to be somewhat higher than it is for peptides hydrolyzed in the absence of carbohydrates. However, results of destruction studies (Section IV) indicate that the destruction of amino acids during hydrolysis of these glycopeptides was neglibible.

b. Carbohydrate Composition.

(i) Qualitative Analysis and the Distribution of Hexoses and Fucose. The identification of the carbohydrate components of each of the glycopeptides was achieved by paper chromatography. Aliquots of the stock solutions were hydrolyzed with dilute sulfuric acid, and the hydrolysates were prepared for chromatography by the procedure employed for the identification of the component sugars of the intact γ-globulin (Section V-A-1), except that the barium sulfate and barium carbonate precipitates were removed by filtration of the hydrolysate through a fine sintered glass funnel. Further removal of non-carbohydrate material was not necessary, and the filtrate was then concentrated to a convenient volume for chromatography. Chromatography was performed in a n-butanol-pyridine-water (6:4:3) system with the use of the multiple development technique, and the chromatograms were sprayed with p-anisidine phthalate reagent (Section III-B-1). The relative distribution of hexoses and fucose was estimated from the freshly developed chromatograms by the method described in Section III-B-2.

Each of the glycopeptides was found to contain all of the sugars, with the possible exception of sialic acid, found in the intact γ-globulin (Section V-A-1), namely, glucosamine, galactose, mannose and fucose. A qualitative test for sialic acid was not performed on the glycopeptides, but the results of the photometric analysis for sialic acid, below, indicate its presence in the glycopeptides. In addition to the above carbohydrate components, a small amount of glucose was detected in each glycopeptide preparation. Since glucose could not be detected in the intact γ-globulin, it must have been present in the glycopeptide preparation as a contaminant. The relative number of residues of galactose, mannose,

fucose and glucose appeared to be the same in each of the glycopeptides and was estimated to be 3, 5, 2 and 1, respectively.

(ii) Quantitative Analysis. The results of the quantitative carbohydrate analyses are tabulated in Tables 3, 4 and 5. Details of the analytical procedures are given below.

Hexose. The hexose contents of the glycopeptides were determined by the orcinol-H_2SO_4 method (Section III-B-3-a). The analyses were performed on duplicate 0.20 ml aliquots (approximately 0.4 mg) of the stock solutions. The values obtained for glycopeptides 1, 2 and 3 are 2.23 $\pm$ 0.01, 2.40 $\pm$ 0.02 and 2.61 $\pm$ 0.02 mmoles of 1:1 galactose-mannose equivalents per gram of glycopeptide, respectively. When corrected for fucose content (below) and the 3 to 5 ratio of galactose to mannose, these values become 1.95, 2.07 and 2.33 mmoles of hexose (galactose and mannose) per gram of glycopeptide, respectively. These values have not been corrected for the glucose contaminant, but, since galactose, mannose and glucose are present in the approximate ratio of 3:5:1 (Section V-A-3-b-(i)) and the color yield of glucose is only 38% of that of 3:5 galactose-mannose (Table 1), the glucose introduces only a small error.

Hexosamine. The hexosamine contents of the three glycopeptides were determined in conjunction with the quantitative amino acid analyses described in Section V-A-3-a. A single hexosamine peak emerged from the analyzer column, and the hexosamine was identified as glucosamine by paper chromatography (Section V-A-1). The values obtained, and tabulated in Tables 3, 4 and 5, are not corrected for destruction during hydrolysis. The results of destruction studies (Section IV) indicate that glucosamine is destroyed to the extent of about 45 to 50% under these conditions.

Fucose. The fucose contents of glycopeptides 1, 2 and 3, as determined by the method of Shettles and Dische (Section III-B-3-c), were found to be 0.46 ± 0.01, 0.52 ± 0.00 and 0.54 ± 0.00 mmole/g of glycopeptide, respectively. The analyses were performed on duplicate sample pairs (0.10 ml of stock solution). Cysteine was omitted from one of each sample pair to correct for non-specific coloring.

Sialic Acid. The glycopeptides were assayed for sialic acid by the direct Ehrlich method of Werner and Odin, as described in Section III-B-3-d. The analyses were performed on duplicate 0.5 ml aliquots of the stock solutions (approximately 1 mg). The values obtained for glycopeptides 1, 2 and 3 are 0.25 ± 0.01, 0.15 ± 0.02 and 0.03 ± 0.00 mmole/g of glycopeptide, respectively. The sialic acid content was calculated as N-acetylneuraminic acid since this is the only type of sialic acid that has been detected in human blood plasma (25).

c. Correlation of Analytical Data. The molecular weights of the three glycopeptides were estimated from their chemical compositions by averaging the weights calculated for each component to contain a whole number of residues (Tables 3, 4 and 5, column 4). Glucosamine was calculated as the N-acetyl derivative (79), and, as mentioned previously, sialic acid was calculated as N-acetylneuraminic acid. The average molecular weights so obtained are 4542, 4648 and 3818 for glycopeptides 1, 2 and 3, respectively. The molecular weights calculated on the basis of those amino acids listed below tyrosine in Tables 3, 4 and 5 were not included in the average since the number of carbohydrate residues calculated on the basis of such large molecular weights would be much greater than the number known to be present in the intact γ-globulin (Section V-A-1). This is especially evident in the

TABLE 3

COMPOSITION OF GLYCOPEPTIDE 1 FROM HUMAN II-3 γ-GLOBULIN

1	2	3	4	5	6
Residue	Millimoles of Residue per Gram of Sample	Grams of Residue per 100 Grams of Sample	Grams Containing Whole No. of Residues	Residues per 4542 Grams of Sample[a]	Estimated No. of Residues per Mole
Hexose	1.95	31.5	4629/9	8.8[b]	8[b]
Glucosamine[c]	1.09	24.2	4591/5	5.0	5
Fucose	0.46	6.7	4362/2	2.1	2
Sialic Acid[d]	0.25	8.3	4015/1	1.1	1
Aspartic Acid	0.60	6.9	5004/3	2.7	3
Glutamic Acid	0.88	11.4	4531/4	4.00	4
Tyrosine	0.22	3.5	4662/1	0.97	1
Phenylalanine	0.15	2.1	7009/1	0.65	-
Threonine	0.11	1.1	9192/1	0.49	-
Serine	0.17	1.5	5806/1	0.78	-
Valine	0.12	1.2	8262/1	0.55	-
Totals		98.4			24

[a] This molecular weight is the average of the weights containing a whole number of residues (Column 4). The weights calculated from the amino acids listed below tyrosine are not included in the average (Section V-A-3-c).

[b] Estimated as 8 rather than 9 hexose residues because of contamination of the glycopeptide preparation with an amount of glucose equivalent to approximately 1 residue (Section V-A-3-b-(i)).

[c] Calculated as N-acetylglucosamine. The value listed is not corrected for destruction during acid hydrolysis.

[d] Calculated as N-acetylneuraminic acid.

TABLE 4

COMPOSITION OF GLYCOPEPTIDE 2 FROM HUMAN II-3 γ-GLOBULIN

1	2	3	4	5	6
Residue	Millimoles of Residue per Gram of Sample	Grams of Residue per 100 Grams of Sample	Grams Containing Whole No. of Residues	Residues per 4648 Grams of Sample[a]	Estimated No. of Residues per Mole
Hexose	2.07	33.5	4836/10	9.6[b]	9[b]
Glucosamine[c]	1.04	23.1	4846/5	4.8	5
Fucose	0.52	7.7	3796/2	2.4	2
Sialic Acid[d]	0.15	4.9	6802/1	0.7	1
Aspartic Acid	0.45	5.1	4514/2	2.06	2
Glutamic Acid	0.59	7.6	5097/3	2.74	3
Tyrosine	0.21	3.4	4800/1	0.97	1
Phenylalanine	0.14	2.1	7009/1	0.66	-
Threonine	0.03	0.3	33707/1	0.13	-
Serine	0.08	0.7	12442/1	0.37	-
Totals		88.4			22-23

[a] See footnote "a" of Table 3.

[b] Estimated as 9 rather than 10 hexose residues because of contamination of the glycopeptide preparation with an amount of glucose equivalent to approximately 1 residue (Section V-A-3-b-(i)).

[c] See footnote "c" of Table 3.

[d] See footnote "d" of Table 3.

TABLE 5

COMPOSITION OF GLYCOPEPTIDE 3 FROM HUMAN II-3 γ-GLOBULIN

1	2	3	4	5	6
Residue	Millimoles of Residue per Gram of Sample	Grams of Residue per 100 Grams of Sample	Grams Containing Whole No. of Residues	Residues per 3818 Grams of Sample[a]	Estimated No. of Residues per Mole
Hexose	2.33	37.7	3869/9	8.9[b]	8[b]
Glucosamine[c]	0.82	18.2	3657/3	3.1	3
Fucose	0.54	7.8	3744/2	2.0	2
Sialic Acid[d]	0.03	1.0	33330/1	0.1	-
Aspartic Acid	0.49	5.6	4090/2	1.87	2
Glutamic Acid	0.27	3.5	3692/1	1.03	1
Tyrosine	0.26	4.2	3859/1	0.99	1
Phenylalanine	0.06	0.8	19940/1	0.19	-
Threonine	0.03	0.3	-	-	-
Serine	0.06	0.5	-	-	-
Alanine	0.02	0.1	-	-	-
Valine	0.02	0.2	-	-	-
Glycine	0.03	0.2	-	-	-
Totals		80.1			17

[a] This molecular weight is the average of the weights containing a whole number of residues (Column 4). The weights calculated from sialic acid and those amino acids listed below tyrosine are not included in the average (Section V-A-3-c).

[b] See footnote "b" of Table 3.

[c] See footnote "c" of Table 3.

[d] See footnote "d" of Table 3.

data on glycopeptide 3 (Table 5), in which the quantities of the amino acids listed below tyrosine are much smaller than the quantity of tyrosine. The number of residues of each component (Column 5) was calculated from the average molecular weight, and from these values the number of whole residues of each component (Column 6) was estimated. The molecular weights calculated from the estimated number of whole residues are 4058, 3976 and 2778 for glycopeptides 1, 2 and 3, respectively. The complete analytical compositions given in the tables represent 98.4%, 88.4% and 80.1% and the amino acids present as impurities account for 5.9%, 3.1% and 2.1% of the weights of glycopeptide preparations 1, 2 and 3, respectively. Since correction was not made for destruction of amino acids and glucosamine during acid hydrolysis and for the moisture content of the glycopeptide precipitates, the recovery values indicate that all of the components of the glycopeptides have probably been accounted for.

d. Amino-terminal Amino Acid Analysis. End group analysis of glycopeptides 1 and 3 was performed on 0.5 ml aliquots of the stock solutions (approximately 1 mg) by the fluorodinitrobenzene (FDNB) method of Sanger (95,96). The dinitrophenylation reaction was performed in an ethanol-trimethylamine medium, and the DNP-glycopeptides were isolated and hydrolyzed with HCl essentially according to the procedure of Levy (97). The acid hydrolysates were extracted with ether and the ether-extractable components were separated by partition chromatography on celite columns as described by McFadden and Smith (21) and Thompson (98). From the chromatographic behavior of the DNP-amino acids on the celite clolumns, the amino-terminal amino acids of glycopeptides 1 and 3 appeared to be glutamic acid

and aspartic acid, respectively. Only one DNP-amino acid was detected in each case. The identification of glutamic acid as the amino-terminal amino acid of glycopeptide 1 was confirmed by chromatography of the DNP-amino acid isolated on the celite column on Whatman No. 4 paper in a t-amyl alcohol-phthalate buffer system (97). The DNP-amino acid from glycopeptide 3 was not isolated in sufficient quantity to permit confirmation. Lack of a sufficient quantity of glycopeptide 2 prevented end group analysis of this glycopeptide.

B. Summary and Conclusions

1. Composition of Glycopeptides. The compositions of the three glycopeptides isolated from a papain hydrolysate of human II-3 γ-globulin have been summarized in Table 6. The number of residues of each component was estimated from the analytical compositions alone since no molecular weight determinations were performed. Calculated on the basis of the compositions listed in Table 6, the molecular weights of glycopeptides 1, 2 and 3 are 4058, 3976 and 2778, respectively. The total analytical compositions of glycopeptides 1, 2 and 3 (Tables 3, 4 and 5) account for 98.4%, 88.4% and 80.1% of the weights of the glycopeptide preparations, respectively. Since the results of the amino acid and glucosamine analyses were not corrected for destruction during acid hydrolysis and the moisture content of the glycopeptide precipitates was not taken into consideration in calculating recoveries, it is probable that all of the components of the glycopeptides have been accounted for.

The carbohydrate compositions of glycopeptides 1 and 2 are the same, except glycopeptide 2 appears to contain somewhat less sialic acid than glycopeptide 1. These differ from glycopeptide 3 in that the latter has

TABLE 6

COMPOSITIONS OF GLYCOPEPTIDES FROM HUMAN II-3 AND II-1,2 γ-GLOBULINS

Residue	Estimated Number of Residues					
	II-3 γ-Globulin			II-1,2 γ-Globulin[a]		
	Glycopeptide 1	Glycopeptide 2	Glycopeptide 3	Glycopeptide 1	Glycopeptide 2	Glycopeptide 3
Hexose	8	9	8	8	8	8
Glucosamine[b]	5	5	3	6	6	3
Fucose	2	2	2	2	2	2
Sialic Acid[c]	1	1 (0.7)	0 (0.1)	1	0-1 (0.6)	0-1 (0.2)
Aspartic Acid	3	2	2	2	2	2
Glutamic Acid	4	3	1	3	2	1
Tyrosine	1	1	1	1	1	1
Totals	24	22-23	17-18	23	21-22	17-18

[a] From the data of Rosevear (60).

[b] The glucosamine values are not corrected for destruction during acid hydrolysis. (See Section V-B-3).

[c] The values in parentheses are the actual analytical values obtained.

fewer glucosamine residues and little or no sialic acid. Within the limits of accuracy of the analytical methods employed for their determination, the number of residues of hexose and fucose is the same in all three glycopeptides, and the hexose consists of galactose and mannose in the approximate molar ratio of 3 to 5 in each case. The amino acid compositions of the three glycopeptides differ only in the number of aspartic acid and glutamic acid residues. The possible significance of the differences in the compositions of the glycopeptides is discussed below.

2. Comparison of Glycopeptides from Human II-3 and Human II-1,2 γ-Globulin. Included in Table 6 for comparison with the three glycopeptides isolated from human II-3 γ-globulin are the compositions of three glycopeptides isolated from papain hydrolysates of human II-1,2 γ-globulin, as reported by Rosevear and Smith (29). The glycopeptides from each fraction are numbered in order of their emergence from a starch electrophoresis column. The starch column elution pattern of the glycopeptides from the II-3 fraction, shown in Figure 1, is essentially identical to that of the glycopeptides from II-1,2 γ-globulin (29); the positive pole was at the bottom of the column in each case.

It can be seen from Table 6 that the carbohydrate compositions of the glycopeptides from the II-3 γ-globulin are in good agreement with the compositions of the corresponding glycopeptides from the II-1,2 fraction. The hexose of each of the glycopeptides is made up of galactose and mannose in the approximate molar ratio of 3 to 5. Although the number 3 glycopeptides are the same with respect to both carbohydrate and amino acid composition, the analytical data indicate that glycopeptides 1 and 2 from the II-3 fraction have one more residue each of aspartic acid and glutamic acid than

the corresponding glycopeptides from II-1,2 γ-globulin. However, the presence of appreciable quantities of contaminating amino acids in these two glycopeptides from the II-3 fraction (Tables 3 and 4) suggest that the aspartic acid and glutamic acid values may be elevated due to contamination.

The following amino acid sequences have been reported (29) for the glycopeptides isolated from human II-1,2 γ-globulin:

Glycopeptide 1: Glu.Glu.Asp-NH_2.Tyr.Glu.Asp.(Carbohydrate)

Glycopeptide 2: Glu.Asp-NH_2.Tyr.Glu.Asp.(Carbohydrate)

Glycopeptide 3: Asp.Tyr.Glu.Asp.(Carbohydrate)

The carbohydrate moieties were shown to be attached to the asparty1 residues, as indicated, and it was suggested that the attachment is by way of an amide or ester bond involving the β-carboxyl group of the asparty1 residue. In the present study of glycopeptides from II-3 γ-globulin, end group analyses on glycopeptides 1 and 3 indicate that a glutamyl residue is amino-terminal in the former and an asparty1 residue in the latter, as in the corresponding glycopeptides from II-1,2 γ-globulin.

Thus, although the analytical data indicate that the amino acid compositions of glycopeptides 1 and 2 of the II-3 γ-globulin are somewhat different from the corresponding glycopeptides from the II-1,2 fraction, the carbohydrate compositions, together with the results of the end group analyses and the highly similar starch column elution patterns, strongly indicate that the corresponding glycopeptides from these two γ-globulin fractions are identical. The lack of sufficient quantities of the glycopeptides prevented the attainment of more conclusive proof. That the carbohydrate compositions of these protein fractions are the same is further supported by the finding of Smith *et al.* (84) that their total hexose and

total hexosamine contents are the same. Final proof of the identity of the carbohydrate moieties awaits elucidation of the monosaccharide sequences.

3. Comparison of the Carbohydrate Compositions of the Glycopeptides and the Intact II-3 γ-Globulin. Qualitative analysis has shown that glycopeptides 1 and 2 contain all of the carbohydrate species found in the intact γ-globulin, i.e., glucosamine, galactose, mannose, fucose and sialic acid. Qualitatively, glycopeptide 3 differs in its carbohydrate composition only in that it lacks sialic acid. The intact γ-globulin has been found to contain 7 to 8 residues of hexose; thus, the hexose content of each of the three glycopeptides, which were found to contain about 8 residues of hexose, accounts for all of the hexose found in the intact glycoprotein. The hexosamine content (as glucosamine) of the same preparation of II-3 γ-globulin was found by Smith et al. (84), using a modification of the procedure of Elson and Morgan, to be 1.23%, which is equivalent to about 12 residues of glucosamine. The glucosamine content of glycopeptides 1 and 2, as determined in conjunction with amino acid analyses by the ion-exchange chromatography procedure of Moore, Spackman and Stein (Section V-A-3-a) is equivalent to only 5 residues and that of glycopeptide 3 to only 3 residues. However, since these analyses were performed, experiments have been conducted (Section IV) which indicate that the extent of glucosamine destruction under the conditions of the analytical procedure followed is of the order of 45 to 50%. If this destruction factor can be applied in this case, the glucosamine contents of glycopeptides 1, 2 and 3 become approximately 10, 10 and 6 residues, respectively, and, in the case of the glycopeptides from II-1,2 γ-globulin, the glucosamine content of which was determined in the same manner, the corrected values are 12,

12 and 6 residues, respectively. This would indicate that the glucosamine contents of glycopeptides 1 and 2 account for all of the glucosamine in the intact protein molecule. These considerations, together with the apparent over-lapping of amino acid sequences, give strong support to the conclusion that glycopeptides 1 and 2 arose from the same structure in the intact glycoprotein. Although glycopeptide 3 differs from the others in that it has less sialic acid and glucosamine and contains an aspartyl residue where the others have an asparaginyl residue, if glycopeptides 1 and 2 each account for all of the carbohydrate of the intact glycoprotein, it follows that glycopeptide 3 must have arisen from the same locus in the protein and that the sialic acid, the amide group of the asparaginyl residue and part of the glucosamine residues were lost during preparation of the glycopeptides.

Thus, it appears, as was concluded by Rosevear and Smith (29) in the case of human II-1,2 γ-globulin, that human II-3 γ-globulin contains a single carbohydrate moiety which is linked by a covalent bond to an aspartyl residue in the protein.

VI. RABBIT γ-GLOBULIN AND ANTIBODIES

A. Experimental Methods and Results

1. Carbohydrate Composition of Rabbit γ-Globulin. Carbohydrate analyses were performed on γ-globulin prepared from normal rabbit serum by the ion-exchange cellulose chromatography method of Sober et al. (12). Only the column effluent fractions corresponding to the major peak, which obviously contained more than one component, were pooled for use. This preparation behaved as a homogeneous substance during electrophoresis for 300 minutes in 0.1 N Veronal buffer, pH 8.5, with a potential gradient of 5.9 volts/cm. The electrophoretic mobility, based on a single determination under the above conditions, was 1.8×10^{-5} cm^2/volt/sec. The electrophoretic studies were performed with a Spinco Model H moving boundary electrophoresis apparatus.

a. Qualitative Analysis and the Distribution of Hexoses and Fucose. Paper chromatography was employed for identification of the component carbohydrates. Samples were hydrolyzed with dilute sulfuric acid, and the hydrolysates were prepared for chromatography as described in Section V-A-1. Aliquots of the hydrolysates were chromatographed in n-butanol-pyridine-water (6:4:3) and in sec-butanol-acetone-acetic acid-water (3:3:1.5:2.5). The orcinol-TCA and thiobarbituric acid spray reagents were used for the detection of sialic acid; other sugars were detected with the p-anisidine phthalate and basic silver reagents (Sec-

tion III-B-1). By comparison with authentic sugars chromatographed with the hydrolysates, the carbohydrate components of rabbit γ-globulin were identified as glucosamine, galactose, mannose, fucose and sialic acid, as in the case of human γ-globulin. The molar ratios of galactose, mannose and fucose residues, estimated from the chromatograms as described in Section III-B-2, are approximately 2 to 4 to 1.

b. Quantitative Analysis. For quantitative sugar analysis, a stock solution of the purified γ-globulin was prepared. The γ-globulin was dissolved in 0.9% NaCl and dialyzed against this solvent at 5°. The protein solution was filtered through a sintered glass funnel to remove any lint and other insolubles that may have been present. The protein nitrogen concentration of the stock solution was determined as described in Section III-C, and the protein concentration (53.2 mg/ml) was calculated from the nitrogen concentration, based on a nitrogen content of rabbit γ-globulin of 16% (99).

Hexose. Duplicate 0.5 ml (26.6 mg) aliquots of stock solution were analyzed for hexose by the photometric orcinol-sulfuric acid method. The absorbancies were corrected for nonspecific color as previously described (Section III-B-3-a). The total hexose value obtained is 0.71 ± 0.01%, or 7.0 moles of 1:1 galactose-mannose equivalents per mole of protein. When this value is corrected for fucose (below) and the ratio of galactose to mannose, as described in Section III-B-3-a, it becomes 0.67% or 6.6 moles of hexose per mole of protein. These results indicate the presence of a total of 6 to 7 residues of galactose and mannose per γ-globulin molecule.

Fucose. Fucose analysis of duplicate 0.3 ml (16.0 mg) aliquots of stock solution by the method of Dische and Shettles (Section III-B-3-c)

indicated the fucose content of rabbit γ-globulin to be 0.05 ± 0.00%. This is equivalent to only 0.56 mole of fucose per mole of protein. A check of this analysis produced the same results within the limits of accuracy. The results indicate there is probably 1 residue of fucose per protein molecule, but the possibility that protein which does not contain fucose is present in the γ-globulin preparation cannot be dismissed. Dische and Shettles (75) have shown that the presence of protein and hexose may decrease the net color yield from fucose in this determination. However, in the cases studied by these investigators, the interference was only slight.

Sialic Acid. Rabbit γ-globulin was found by the thiobarbituric acid assay method (Section III-B-3-d) to contain 0.12 ± 0.00% sialic acid (as N-acetylneuraminic acid), which is equivalent to 0.60 mole per mole of protein. The analysis was performed on duplicate 0.2 ml (10.6 mg) aliquots of the stock solution. Interference with this determination by the small amount of fucose present (above) was found to be negligible and was not corrected for.

2. Inert γ-Globulin and Specific Antibody From Type VII Antipneumococcus Serum. To characterize further the carbohydrate of rabbit γ-globulin, a γ-globulin preparation from rabbit Type VII Pneumococcus antiserum was studied. This same preparation was reported by McFadden and Smith (21) to contain 87% γ-globulin and 3 to 4% serum albumin; the remainder is made up of α- and β-serum globulins. The specific antibody was found to account for 14% by weight of the γ-globulin. In an attempt to compare the nature of the carbohydrate of the specific antibody and the inert γ-globulin, the antibody was separated from the remainder of the protein by precipitation with the specific antigen (Type VII Pneumococcus capsular polysaccharide),

the inert γ-globulin and antigen-antibody precipitate were digested with papain, and the fractionation procedure employed for isolating glycopeptides from human γ-globulin (Section V-A-2) was applied to the isolation of glycopeptides from these fractions. In the following discussion, the Type VII Pneumococcus antibody will be referred to simply as the antibody fraction and that fraction of the γ-globulin which was not precipitable with the specific antigen will be referred to as "inert" γ-globulin, although, presumably, this fraction contains antibodies to other antigens (Section I).

a. Precipitation of the Specific Antibody. A solution of Type VII Pneumococcus capsular polysaccharide in 0.9% NaCl (approximately 2 mg/ml) was added to a 4% solution of the γ-globulin preparation in 0.9% NaCl until the equivalence point was reached. The equivalence point is the point at which neither free antibody nor antigen remains in solution. This point was reached by the cautious addition of antigen until no further precipitation was observed. The antigen was added intermittently over a period of several hours, and the precipitate was allowed to settle out in the cold between each addition. The resultant antigen-antibody precipitate was collected by centrifugation and washed with 0.9% NaCl. The precipitate was suspended in water, and the protein was denatured by heating the suspension in a water bath at 75° to 85° with stirring. The temperature was maintained within this range for 20 minutes. Photometric hexose determinations indicated that some, but not all, of the antigen polysaccharide was released into solution from the antigen-antibody complex by the heat denaturation. The precipitate was collected by centrifugation, washed with water, absolute ethanol and, finally, anhydrous

ether, and dried in vacuo over phosphorous pentoxide.

The inert γ-globulin was precipitated from the supernatant solution by heat denaturation, and the precipitate was collected, washed and dried as described above.

b. Preparation of Glycopeptides. Twenty grams of the heat-denatured inert γ-globulin and 21 g of heat-denatured antigen-antibody precipitate, each suspended in 200 ml of water, were digested with papain under the same conditions and enzyme-substrate ratios as described for the digestion of human γ-globulin (Section V-A-2). The mercuripapain employed in this instance had a C_1 of 1.2. In each case, the digestion appeared complete after 25 to 26 hours, but incubation was continued for an additional 24 hours to insure complete digestion. A small quantity of insoluble material, which was found to be free of carbohydrate, was removed from each of the digestion mixtures by centrifugation, and the clear supernatant solutions were fractionated by passing them through columns (2.2 cm x 25 cm) of Dowex 50-X8 resin in the hydrogen cycle and precipitation of the carbohydrate-rich material in the resin column effluents from 92.5% ethanol as described in steps 2 and 3 of Section V-A-2.

Conditions for further purification by starch column electrophoresis (step 4) were determined by electrophoresis of portions of the precipitates obtained in step 3 on a Spinco Model R electrophoresis apparatus, as before. In each case, the only detectable carbohydrate migrated toward the negative pole in pH 8.6 Veronal buffer. This is in contrast to the glycopeptides isolated from human γ-globulin, all of which bore a net negative charge at this pH. To permit detection of carbohydrate, electrophoresis was performed on fiber glass strips (3 cm x 30 cm), and, following "fixa-

tion" by heating the strips at 105° for 30 minutes, the strips were dipped in Molisch reagent (0.5 g α-naphthol, 20 ml concentrated sulfuric acid and 480 ml absolute ethanol). In the case of the inert γ-globulin preparation, 1 mg portions of the precipitate dissolved in 0.01 ml of buffer were applied to the fiber glass strips. Electrophoresis was performed in 0.05 N Veronal buffer, pH 8.6, for 20 1/2 hours with an initial potential of 290 volts and a constant current of 0.6 ma per strip. The only detectable carbohydrate migrated toward the negative pole as a single, diffuse band. The ninhydrin-positive material on these strips was very diffuse, and no conclusions as to its nature could be drawn. By electrophoresis of the sample on paper strips under the same conditions, it was found that nearly all of the ninhydrin-positive material migrated toward the positive pole; only 3 bands which, like the carbohydrate, migrated toward the negative pole were detected with ninhydrin. One of these had a relatively high mobility, while the other two moved just off the origin and were poorly resolved. From the starch column electrophoresis elution diagram (Figure 2) it appears these two slower moving components were glycopeptides.

In the same manner, it was found that nearly all of the ninhydrin-positive material in the antibody preparation from step 3 migrated toward the positive pole, whereas the only detectable carbohydrate migrated as a single, broad band toward the negative pole.

These preliminary experiments established the conditions for further purification of the glycopeptide preparations by starch column electrophoresis. The inert γ-globulin glycopeptide preparation from step 3 was submitted to starch column electrophoresis in 200 to 275 mg portions. The

electophoresis was performed for 78 to 80 hours under the conditions previously described (Step 4, Section V-A-2), except in this instance the negative electrode was placed at the bottom of the starch column and the electrophoresis apparatus employed was designed after that described by Flodin and Kupke (91), in which the starch column is placed in a cylinder of buffer to insure a more uniform temperature during electrophoresis. The starch column was eluted with buffer and the effluent collected in approximately 0.5 ml fractions, as before. Equal aliquots (0.075 ml) of every fifth fraction were assayed for hexose by the orcinol-sulfuric acid method (Section III-B-3-a) and for free amino groups by a photometric ninhydrin procedure (Section III-A-2). An elution diagram obtained by plotting the absorbancies against the fraction number is shown in Figure 2. It may be noted that, in contrast to the glycopeptides isolated from human γ-globulin, the orcinol color yields are much higher than the ninhydrin color yields from a given aliquot. Since the slower migrating of the two components shown in Figure 2 was present in only small amounts (less than 10% of the total carbohydrate recovered), it was not studied further. The fractions corresponding to the larger peak were pooled as indicated in the figure. These fractions from three such electrophoresis runs were combined, the glycopeptide was precipitated from 92.5% ethanol, and the precipitate was washed and dried in the manner previously described.

In a preliminary starch column electrophoresis run under conditions identical to those described above, the carbohydrate component of the antibody preparation from step 3 emerged from the column as a single, broad, unsymmetrical peak and was badly contaminated with ninhydrin-positive material. Much of the contaminating ninhydrin-positive material was removed by

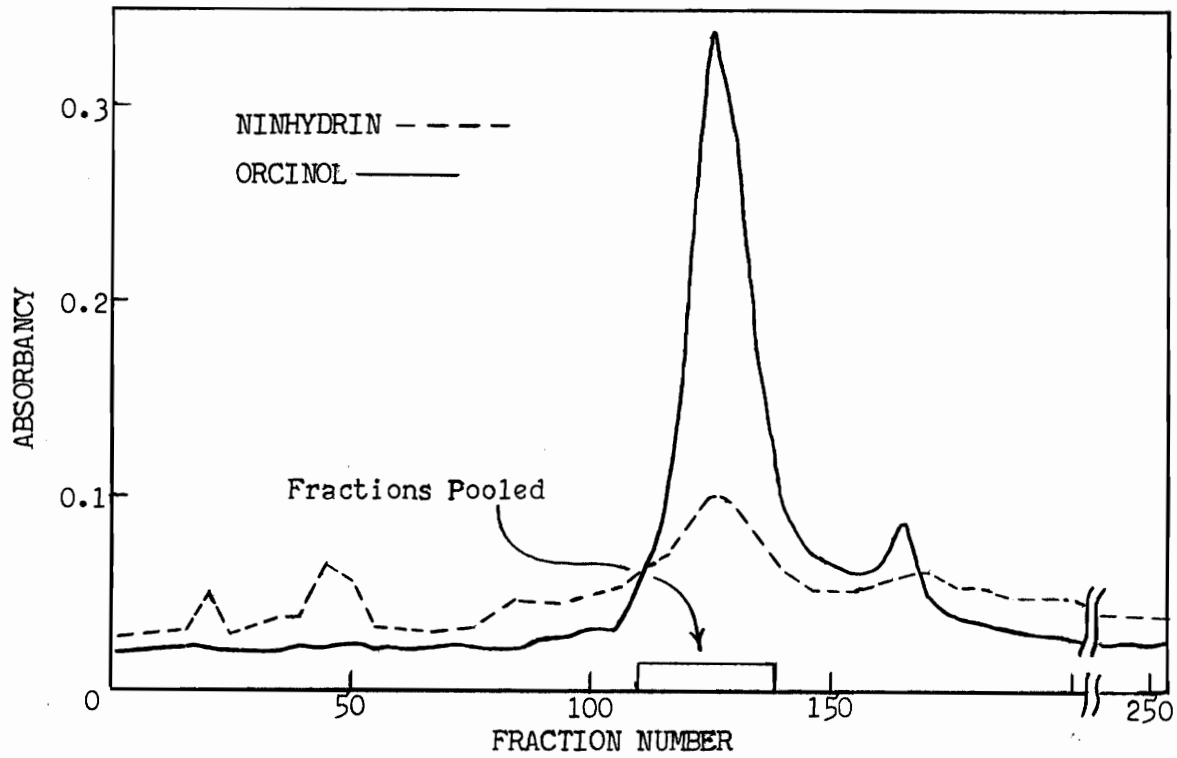

Figure 2. Starch column electrophoresis elution diagram of a glycopeptide from "inert" rabbit γ-globulin. Electrophoresis was performed in 0.05 M Veronal buffer, pH 8.6. Effluent fractions contained 0.5 ml each.

re-running the preparation through steps 2 and 3. This procedure did not, however, improve the resolution of the carbohydrate-containing components. The precipitate from step 3 was submitted to starch column electrophoresis in 150 mg portions under the conditions described above. The precipitate was found to be much less soluble than the corresponding fraction from the inert γ-globulin, and, because of its relatively low solubility, larger portions could not be applied to the starch column. The effluent fractions corresponding to the orcinol-positive peak were combined. These fractions from five such runs were pooled, the material was precipitated from 92.5% ethanol and the precipitate washed and dried as previously described.

Preliminary amino acid analyses of the glycopeptide fractions from the

starch column electrophoresis step indicated that further purification was desirable. Therefore, 20 mg each of the glycopeptide preparations from the inert γ-globulin and the antibody fraction were further purified by chromatography on Whatman 3mm paper in a descending n-butanol-acetic acid-water (200:30:75) system for 70 to 72 hours. In each case the glycopeptides were found to migrate only 1 to 2 cm off of the origin. The glycopeptide fractions were eluted from the paper with water, and the eluates were freeze-dried. A number of amino acids present in small amounts in hydrolysates of the glycopeptide preparation from step 4 were removed by this procedure.

Although the recovery of the orcinol-positive material from the papain digests and the Dowex 50 treatment step was nearly quantitative, loss in the subsequent steps reduced the total recovery to the order of 20% in the case of the inert γ-globulin preparation. The recovery of glycopeptides from the antibody fraction could not be estimated for reasons that will become apparent below.

c. Characterization of Glycopeptides. For further study of the glycopeptides, stock solutions of the purified fractions were prepared. The glycopeptide preparations were dissolved in water, and the solutions were filtered through a sintered glass funnel to remove any lint and other insolubles that may have been present and diluted with lint-free water to a final concentration of approximately 2 mg/ml.

(i) Carbohydrate Composition. The component sugars of the glycopeptides were identified by paper chromatography. Aliquots of the stock solutions were hydrolyzed in dilute sulfuric acid and the hydrolysates were prepared for chromatography as described in Section V-A-3-c.

Chromatograms were developed in n-butanol-pyridine-water (6:4:3) with the use of the multiple development technique (Section III-B-2) to obtain the desired resolution. The orcinol-TCA reagent was used for the detection of sialic acids; other sugars were detected with the p-anisidine phthalate reagent (Section III-B-1).

The glycopeptide from the inert γ-globulin fraction was found to contain glucosamine, galactose, mannose and fucose. The molar ratios of galactose, mannose and fucose were estimated from the chromatograms in the manner described in Section III-B-2 to be approximately 1 to 3 to 1. The glycopeptide preparation from the antibody fraction was found to contain these same sugars and, in addition, glucose and rhamnose. No sialic acid was detected in either preparation. The results of a preliminary analysis of Type VII Pneumococcus capsular polysaccharide by Barker (100) indicated the presence of the following sugars: galactose, glucose (?), rhamnose and amino sugar(s). From this it appeared that the glycopeptide from the antibody fraction was contaminated with the Type VII capsular polysaccharide used to precipitate the antibody. This was confirmed by a precipitin test; addition of a portion of this glycopeptide preparation dissolved in 0.9% NaCl to a solution of the γ-globulin fraction from rabbit Type VII Pneumococcus antiserum in the same solvent resulted in precipitation of protein. Partial separation of the glycopeptide from the capsular polysaccharide was obtained by paper chromatography in 80% ethanol. However, the components streaked badly in this solvent and the glycopeptide could not be purified suitably on a preparative scale by this means.

Because the glycopeptide preparation from the specific antibody was contaminated with the polysaccharide antigen, quantitative sugar determin-

ations were performed only on the glycopeptide from the inert γ-globulin fraction. The results of these determinations are tabulated in Table 7. Details of the analytical procedures are given below.

Hexose. The total hexose content of the glycopeptide from the inert γ-globulin, as determined by the orcinol-sulfuric acid method (Section III-B-3-a), is 3.60 ± 0.03 μmoles of 1:1 galactose-mannose equivalents/ml. The analysis was performed on duplicate 0.2 ml aliquots of the stock solution (approximately 0.4 mg of glycopeptide). When this value is corrected for the fucose content (below) and the ratio of galactose to mannose (above) as described in Section III-B-3-a, the hexose concentration (as galactose and mannose) becomes 3.03 μmoles/ml.

Glucosamine. Glucosamine was determined in conjunction with the quantitative amino acid analysis of this glycopeptide, below. The uncorrected value obtained by this method is 2.43 μmoles/ml of stock solution. When corrected for approximately 47% destruction of glucosamine under the conditions of this analysis (Section IV), this value becomes 4.6 μmoles/ml.

Fucose. Fucose assays were run on duplicate 0.1 ml aliquots of stock solution (approximately 0.2 mg of glycopeptide) by the method of Dische and Shettles (Section III-B-3-c). The fucose concentration was found by this method to be 0.75 ± 0.02 μmole/ml.

(ii) Amino Acid Composition. Qualitative amino acid analysis of the glycopeptide preparations was accomplished by paper electrophoresis-chromatography as described in Section III-A-1. Aliquots of the stock solutions were hydrolyzed in 6 N HCl for 20 hours at 105° in sealed glass capillary tubes. The hydrolysates were concentrated to dryness *in vacuo* over NaOH and the residues were dissolved in a convenient volume of

buffer for electrophoresis. The major ninhydrin-positive components of the glycopeptides from both the inert γ-globulin and antibody fractions were found to be glucosamine, glutamic acid, aspartic acid and phenylalanine. Much smaller amounts of other amino acids were also present. The molar ratios of the amino acids appeared to be the same in both preparations. As expected, the relative amount of glucosamine was higher in the antibody glycopeptide than in the glycopeptide from the inert γ-globulin. This is due, at least in part, to the presence of Type VII Pneumococcus capsular polysaccharide in the antibody preparation.

Quantitative amino acid and glucosamine analysis of the inert γ-globulin glycopeptide was performed on an automatic amino acid analyzer as described in Section III-A-2. A 0.75 ml aliquot of stock solution was hydrolyzed in 6 N HCl in the presence of sodium bisulfite for 21 hours under the conditions described in Section V-A-3-b. The hydrolysate was concentrated to dryness in vacuo, and the residue was dissolved in 1.20 ml of 0.2 N sodium citrate buffer, pH 2.2. A 1 ml aliquot of this solution (equivalent to approximately 1.2 mg of glycopeptide) was analyzed for amino acids and glucosamine. The results are tabulated in Table 7.

(iii) Molecular Weight Determination. The molecular weight of the glycopeptide from the inert γ-globulin fraction was estimated to be 3150 ± 5.7% by the sedimentation-equilibrium method of Archibald (101), essentially as described by Schachman (102). The determination was performed on a 1.3% solution of glycopeptide in 0.15 M NaCl with a Spinco Model E Ultracentrifuge. The pH of this solution was 6.5. The glycopeptide preparation used for this determination was that obtained in step 4 (starch column electrophoresis) of the fractionation procedure. As previously

indicated, this preparation is only slightly less pure than that used for the amino acid and sugar analyses.

TABLE 7

COMPOSITION OF A GLYCOPEPTIDE FROM INERT RABBIT γ-GLOBULIN

Residue	Calculated Number of Residues[a]	Estimated Whole Number of Residues
Hexose	3.9	4
Glucosamine[b]	6.0	6
Fucose	1.0	1
Sialic Acid	None	None
Aspartic Acid	0.98	1
Glutamic Acid	1.23	1
Phenylalanine[c]	0.79	1

[a] Calculated as described in Section VI-A-2-(iv).

[b] The glucosamine value was determined in conjunction with the amino acid analysis by the method of Moore, Spackman and Stein and has been corrected for loss due to destruction (Section VI-A-2-c-(i)).

[c] Thr, Ser, Pro and Gly were present in amounts equivalent to 0.07 to 0.08 residues; other amino acids were absent or present in smaller amounts.

(iv) Correlation of Data. The number of residues of each component of the inert γ-globulin glycopeptide listed in Table 7 was estimated from the analytical composition. Since aspartic acid, glutamic acid and phenylalanine are present in the approximate ratio of 1 to 1 to 1, the average of the concentrations of these amino acids in the stock solution was taken as the concentration of glycopeptide. This average value, 0.77 μmole/ml, is very close to the concentration of fucose (0.75 μmole/ml).

Since the results of the fucose assay of intact rabbit γ-globulin indicate the presence of not more than one residue of fucose per protein molecule, no more than one residue of fucose could be present in the glycopeptide, and, therefore, the average of the amino acid concentrations seems to be a good approximation of the concentration of the glycopeptide in the stock solution.

The molecular weight calculated from the analytical composition of the glycopeptide is 2423. This is considerably less than the molecular weight determined by the sedimentation-equilibrium method, above. It is possible that dimerization of the glycopeptide occurred under the conditions employed in the molecular weight determination.

3. Rabbit γ-Globulin From Type VII Pneumococcus Antiserum. Glycopeptides were isolated from the papain hydrolysate of a second preparation of γ-globulin from rabbit Type VII Pneumococcus antiserum. By electrophoretic analysis on a Spinco Model H moving boundary electrophoresis apparatus, this preparation was found to contain approximately 93% γ_2-globulin, 1% γ_1-globulin, 3% α_2-globulin and 3% α_1-globulin. To improve the recovery of glycopeptides from the papain hydrolysate, a fractionation procedure somewhat different from that previously described was developed. In this instance, the specific antibody was not separated from the inert γ-globulin. The hydrolysis and fractionation procedures are described below.

a. Preparation of Glycopeptides. The fractionation was followed by the photometric orcinol-sulfuric acid procedure for hexose assay (Section III-B-3-a) and the photometric ninhydrin procedure of Moore and Stein (55). The recovery of orcinol-positive material from each fractionation

step is given in Table 8.

Step 1: Papain Digestion. Forty grams of the salt-free γ-globulin preparation was suspended in water and denatured by heating in a water-bath at 75-85° with stirring. The temperature was maintained in this range for 20 minutes. The denatured γ-globulin, suspended in 400 ml of water, was digested with papain under the same conditions as previously described (Section V-A-2). The enzyme-substrate ratio was increased by the intermittent addition of mercuripapain during the course of the digestion from an initial ratio of 1:100 to a final ratio of 2:100 by weight, as before. The mercuripapain used had a C_1 of 1.5. Photometric ninhydrin assays indicated the digestion was essentially complete after 29 hours. The digestion mixture was then cooled and centrifuged to remove a small quantity (4.2 g, dry weight) of insoluble material, which was found to contain only 0.4% of the total hexose.

Step 2: Dowex 50 Treatment. The supernatant solution from step 1 was treated with Dowex 50-X8 in the hydrogen cycle on a 2.2 cm x 36 cm column of the resin in the manner previously described (Section V-A-2). The pH of the combined column effluents, 3.8, was immediately adjusted to 7.0 with 1 N LiOH at 5°. The recovery of orcinol-positive material was quantitative, while all but 26% of the ninhydrin-positive material was eliminated. Freeze-drying of the combined effluents yielded 16.5 g of light yellow residue. Since the degree of purification achieved in this step was lower than expected, this residue, dissolved in 175 ml of water, was again treated with Dowex 50-X8 in the same manner. In this instance, a 2.2 cm x 25 cm column of freshly regenerated resin was used. The pH of the combined column effluents, 2.4, was adjusted to 7.0 at 5°,

as before. Recovery of the orcinol- and ninhydrin-positive material from this second column was 97% and 25%, respectively; thus, the over-all recovery was 97% and 6%, respectively. Freeze-drying of the combined column effluents yielded 8.5 g of residue.

Step 3: Fractionation and Desalting by Dextran Gel Filtration. Porath and Flodin (103) have recently introduced the use of cross-linked dextrans for the fractionation and desalting of biological substances. Application of this technique to the purification of a variety of substances of biological origin have been reported (104-108). The dextran gels function as "molecular seives" and, thus, afford separation of substances largely according to molecular size. Factors other than molecular size, however, influence the passage of substances through these gels. The flow of small molecular species through such gels is retarded more than that of substances of greater size. The cross-linked dextran employed in this step was G-25 Sephadex (Pharmacia, Upsala, Sweden), which is reported by the manufacturer to retard the flow of substances having a molecular weight less than 2,000 to 3,000. (The water regain, or g of water/g dry gel, of the commercial preparation was 2.30.)

Prior to use, the commercial preparation was washed several times with water, and the "fines" were removed by decantation. The flow rates of the gel columns are very slow if the "fines" are not removed.

Five-hundred mg of the residue from step 2, dissolved in 3 ml of water, was applied to a 2.8 cm x 122 cm column of G-25 Sephadex which had been equilibrated with water in a refrigerated room (5°). The sample was washed into the gel with small portions of water, and the column was eluted in the cold at the approximate rate of 20 ml/hour. The effluent was collected in

6 to 7 ml fractions with an automatic fraction collector. Aliquots of these fractions were assayed for hexose and free amino groups by the photometric orcinol-sulfuric acid and ninhydrin procedures. The carbohydrate-containing material emerged from the column near column volume as a single, broad, unsymmetrical peak, while much of the ninhydrin-reactive material emerged later. The fractions containing the carbohydrate were combined and concentrated to dryness on a rotary evaporator at 28 to 30°. The recovery of orcinol-positive material was 96%, while about 54% of the ninhydrin-positive material was eliminated. In addition to the removal of part of the contaminating peptides and amino acids, desalting of the preparation was also achieved by this process. Desalting was found to be essential for adequate resolution of the glycopeptide components by chromatography on DEAE cellulose, described in the next step.

Step 4: Chromatography on DEAE Cellulose. The glycopeptide fraction from step 3 was chromatographed on a 2.8 cm x 85 cm column of diethylaminoethyl (DEAE) cellulose in Veronal buffer, pH 8.40, with a linear ionic gradient from 0.005 N Veronal to 0.05 N Veronal over the first 3 liters of eluting buffer. Elution was continued from this point with the 0.05 N Veronal.

The residue from step 3 was dissolved in 3.1 ml of the starting buffer and 3.0 ml of this solution was applied to the column; the remainder was used for recovery studies. The column was eluted at 5° at approximately 60 ml/hour. The column effluent was collected in 20 ml fractions with an automatic fraction collector. Photometric hexose assays were run on 1 ml aliquots of every other fraction; 0.5 ml aliquots of the same fractions

were assayed by a photometric ninhydrin procedure. An elution diagram appears in Figure 3. As can be seen from the figure, nearly all of the glycopeptide components emerged from the column in four fractions, which are designated GL-1, GL-2 plus 3, GL-4 and GL-5, in order of their emergence from the columm and, presumably, in order of increasing net negative charge. These four fractions contained 95% of the orcinol-reactive material applied to the column and account for approximately 3, 37, 40 and 8%, respectively, of the orcinol-positive components of the rabbit γ-globulin preparation, which adds up to a total recovery of 88%. The effluent fractions corresponding to each peak were pooled and concentrated to dryness at 28 to 30° on a rotary evaporator.

The gradient elution apparatus employed in this step consisted of two 2 liter plastic cylinders of the same dimensions which were connected at the bottom by Tygon capillary tubing. The cylinder containing the buffer of lower ionic strength was equipped with a mechanical stirrer to insure adequate mixing with the second buffer and was connected to the DEAE cellulose column by a second outlet at the bottom of the cylinder. The two cylinders were maintained at the same horizontal level during the course of elution. According to Sober and Peterson (11), a linear gradient is obtained with two adjacent vessels of identical cross-section, such as these.

The DEAE cellulose column was prepared by the following procedure. The "fines" were removed from a commercial preparation (Brown Company, New Hampshire) of DEAE cellulose by suspending it in water and decanting repeatedly. The DEAE cellulose was washed three to four times with 1 N NaOH on a filter. It was then washed repeatedly with water until the pH of a water suspension of it reached 9 or 10. The pH of the suspension was adjusted to 8.40 by

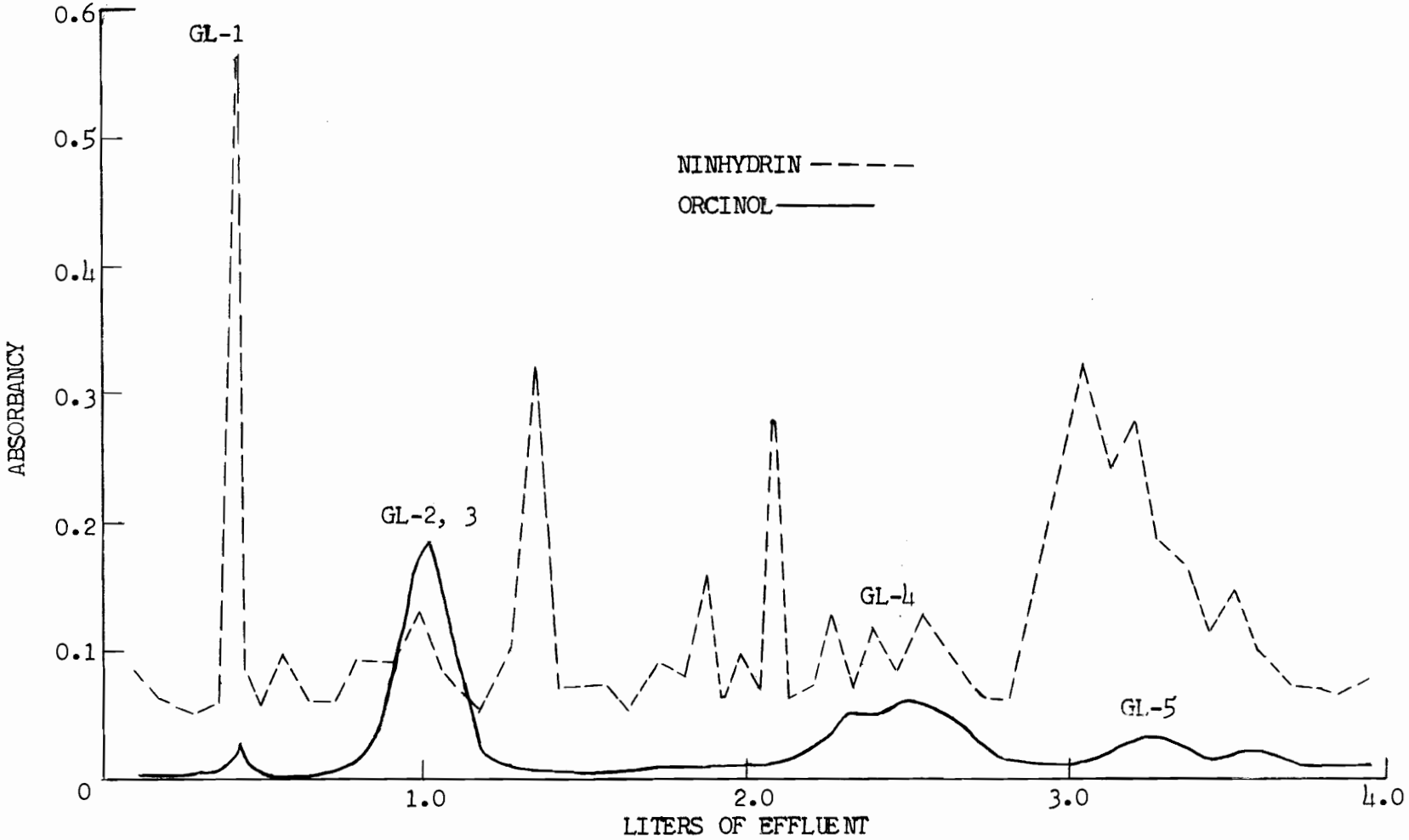

Figure 3. DEAE cellulose column elution diagram of glycopeptides from rabbit γ-globulin. The glycopeptides were eluted with pH 8.4 Veronal buffer with a linear ionic gradient from 0.005 M to 0.05 M Veronal over the first 3 liters of eluting buffer; elution was continued from this point with the 0.05 M Veronal buffer.

the addition of solid barbituric acid. The DEAE cellulose was then washed three times with 0.05 N Veronal buffer, pH 8.40, on a filter, and the column was poured with a suspension of the DEAE cellulose in this buffer. The column was equilibrated against the 0.005 N Veronal at 5° before use. This procedure was adopted when it was found that the cellulose had a marked tendency to aggregate in the more dilute buffer, which resulted in uneven packing of the column.

Step 5: Fractionation and Desalting by Dextran Gel Filtration. Each glycopeptide fraction from step 4 was further fractionated on a 2.8 cm x 122 cm column of G-25 Sephadex. The fractions were eluted from the column with water in the cold as described in step 3. Because of the small quantity of fraction GL-1, the GL-1 fractions from two DEAE cellulose column runs were combined for this step. Due to the low solubility of the Veronal buffer salts present in the fractions from step 4, in applying the fractions to the column, the water was allowed to run below the surface of the Sephadex and the dry sample was applied to the column and carefully packed into a thin, uniform disk on the gel surface. The sample remaining in the sample container was then rinsed on to the column, and the sample was washed into the gel with small portions of water. The column effluent was collected in approximately 8 ml fractions, and 0.5 ml aliquots of the fractions were assayed for hexose and free amino groups, as before. The elution diagrams of glycopeptide fractions GL-2 plus 3, GL-4 and GL-5 appear in Figure 4. Because of the small quantities of fraction GL-1 isolated, it could not be detected in the effluent fractions. However, a knowledge of the effluent volume at which the glycopeptides emerged from the column attained in step 3 permitted recovery of this glycopeptide free of salt, though not free of

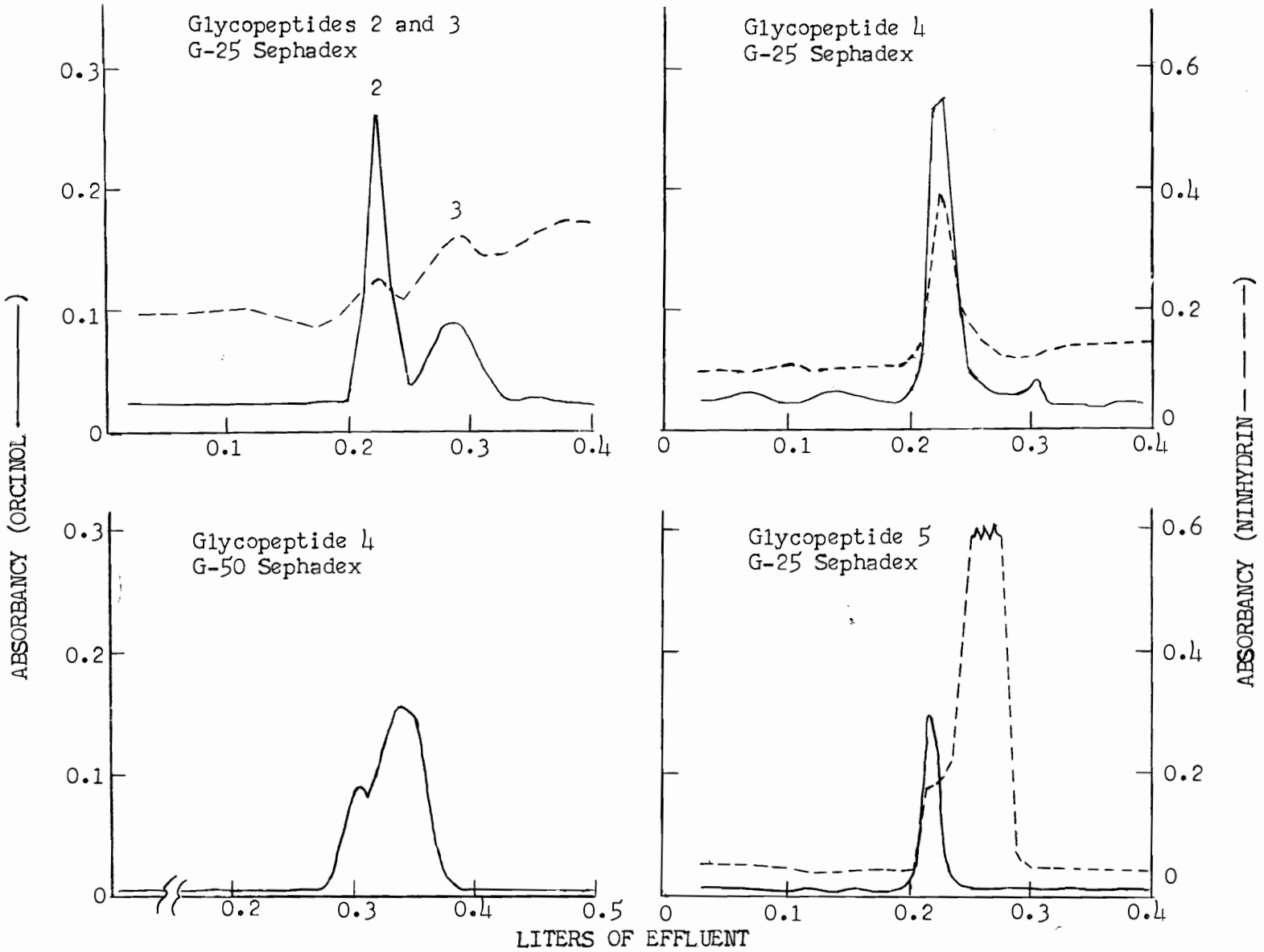

Figure 4. Sephadex column elution diagrams of glycopeptides from rabbit γ-globulin. The columns were eluted with deionized-distilled water.

TABLE 8

RECOVERY OF GLYCOPEPTIDES FROM RABBIT γ-GLOBULIN

Preparation Step	% Recovery (As % of Total Hexose)	
	Each Step	Over-all
Papain Digestion (Step 1)	99	99
Dowex 50 Treatment (Step 2)	97	96
Gel Filtration (Step 3)	96	92
DEAE Column Chromatography (Step 4)	95	88
Gel Filtration (Step 5)	95[a]	83

[a] This value represents the minimum recovery of each of the individual glycopeptide fractions obtained from step 4. The over-all recovery from step 4 (88%) is a better representation of the over-all recovery.

contaminating peptides. Fraction GL-2 plus 3 was separated into two apparently homogeneous fractions, which are designated glycopeptides 2 and 3; glycopeptide 2 emerged at column volume, whereas the movement of glycopeptide 3 was slightly retarded by the Sephadex. The orcinol-positive component of fraction GL-5 emerged as a symmetrical peak, but it was still badly contaminated with ninhydrin-positive substances. Although fraction GL-4 emerged as a single, symmetrical ninhydrin- and orcinol-positive peak, it is obvious from the DEAE cellulose column elution diagram (Figure 3) that it was not homogeneous. Since this fraction emerged from the G-25 Sephadex column at column volume, an attempt was made to fractionate it on a 3 cm x 160 cm column of G-50 Sephadex, which is reported by the manufacturer (Pharmacia, Upsala, Sweden) to retard the

flow of substances having a molecular weight less than 7,000 to 10,000 (water regain = 4.90). The column was prepared as described in step 3, and the sample was eluted from the column with water in the cold (5°) at a flow rate of about 10 ml/hour. The column effluent was collected in 5 ml fractions, and 0.5 ml aliquots were assayed for hexose, as before. The orcinol-positive material emerged as two poorly resolved peaks (Figure 4). Since the minor component emerged first, it is presumably the larger of the two. All of the orcinol-positive fractions were pooled and concentrated to dryness at 28° to 30° on a rotary evaporator. Attempts to separate these two components by paper electrophoresis-chromatography under the conditions described in Section III-A-1 and by paper chromatography in n-butanol-pyridine-acetic acid-water (30:20:6:24, v/v) were unsuccessful. Further purification was not attempted, and the glycopeptide fraction obtained from the G-50 Sephadex column effluent was used for characterization studies. This fraction is designated glycopeptide 4.

Step 6: Further Purification of Glycopeptides 1 and 5. Fraction GL-1 was further purified by paper electrophoresis-chromatography. Two "fingerprints," to each of which was applied one-half of this fraction obtained in step 5, were run simultaneously under the conditions described in Section III-A-1. The location of the components on the "fingerprints" was determined by spraying one of them with ninhydrin reagent. The major ninhydrin-positive component behaved as a positively charged substance under the conditions of electrophoresis. This component was eluted from the second, unsprayed "fingerprint" with water and was found to contain amino acids and glucosamine by spraying a "finger-

print" of the acid hydrolysate (6 N HCl, 105°, 20 hours) with ninhydrin reagent. The composition of this component, designated glycopeptide 1, is discussed in Section VI-B-3-b-(ii).

In the same manner, the glycopeptide component of fraction GL-5 was found to be negatively charged under the conditions of electrophoresis noted above. Further purification of this glycopeptide, designated glycopeptide 5, was achieved by preparative paper electrophoresis in pH 6.4 pyridine-acetate buffer.

b. Characterization of Glycopeptides. For analysis, stock solutions of glycopeptides 2, 3, 4 and 5 were prepared as follows: Each of the glycopeptide preparations from steps 5 and 6, above, was dissolved in a small volume of deionized-distilled water and the solutions were filtered through a medium sintered glass funnel to remove any lint and other insolubles that may have been present. The filtered solutions were diluted with deionized-distilled water (lint-free) to a volume convenient for analysis. The concentrations of the stock solutions of glycopeptides 2, 3, 4 and 5, based on the amino acid concentrations (below), were approximately 0.37, 0.21, 0.68 and 0.18 μmole of glycopeptide per ml. The limited quantity of glycopeptide 1 permitted only qualitative tests on this glycopeptide.

(i) Electrophoresis-Chromatography. Aliquots (approximately 0.1 μmole) of glycopeptides 2, 3 and 4 were submitted to paper electrophoresis-chromatography under the conditions described in Section III-A-1. The electrophoretic and chromatographic conditions were duplicated as nearly as possible in each case. The glycopeptides were detected on the paper with ninhydrin reagent. Each of these three glycopeptides behaved

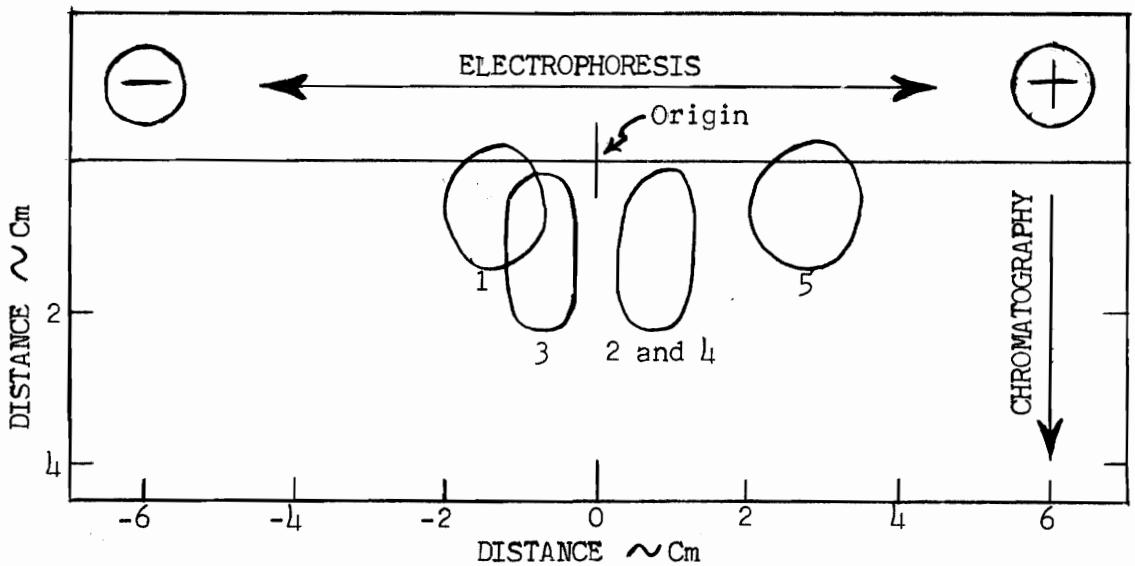

Figure 5. Composite electrophoresis-chromatogram of glycopeptides from rabbit γ-globulin. Electrophoresis was performed in pyridine-acetate buffer, pH 6.4, and chromatography in n-butanol-acetic acid-water (200:30:75, v/v).

as a homogeneous substance under these conditions. It was noted that glycopeptide 2 gave a very weak ninhydrin test and was detected with this reagent only after heating the sprayed chromatogram for several minutes in an oven at 105°.

The behavior of glycopeptides 1 and 5 under the same electrophoretic and chromatographic conditions has been discussed in step 6 of the fractionation proceudre, above. A composite electrophoresis-chromatogram, Figure 5, shows the relative electrophoretic mobilities and R_f values of the five glycopeptides. Glycopeptide 3 behaved as a neutral substance in the pyridine-acetate buffer (pH 6.4). (In paper electrophoresis, neutral substances migrate just off of the origin in the direction of the negative pole due to endosmosis (109).) It would appear that the net charges of glycopeptides 1, 2, 3, 4 and 5 are +1, -1, 0, -1 and -2, respectively.

(ii) Amino Acid Composition. Qualitative amino acid analysis of the glycopeptides was achieved by paper electrophoresis-chromatography of acid hydrolysates of the stock solutions (approximately 0.1 μmole of glycopeptide) as previously described (Section III-A-1). In the case of glycopeptide 1, all of the available preparation from step 6 of the fractionation procedure was used. Hydrolysis was performed in 6 N HCl in sealed capillary tubes for 20 hours at 105°. The hydrolysates were concentrated to dryness in vacuo over NaOH, and the residues were dissolved in a convenient volume of buffer for electrophoresis. The electrophoresis-chromatograms were sprayed with ninhydrin reagent. The major ninhydrin-positive components of the hydrolysates of glycopeptides 2, 3 and 4 were found to be glucosamine, aspartic acid, glutamic acid and phenylalanine; other amino acids were either absent or present in only trace amounts. The major ninhydrin-positive components of glycopeptide 5 were identified as glucosamine and aspartic acid. Other amino acids, including glutamic acid, were present in the preparation, but in much smaller amounts. Phenylalanine was not detected. Glycopeptide 1 was found to contain glucosamine, >2; glutamic acid, 2; aspartic acid, phenylalanine, arginine, leucine, and threonine, 1 each; lysine, 0 to 1; and serine and/or glycine, 1 to 2. The number following each component is an approximation of the relative number of residues, estimated from the size and intensity of the spots. Other amino acids were not detected.

Quantitative amino acid analyses were performed with an automatic amino acid analyzer as described in Section III-A-2. Aliquots of the stock solutions were concentrated to dryness in hydrolysis tubes on a rotary evaporator, and the residues were hydrolyzed in 3 ml of 6 N HCl in sealed,

evacuated tubes. Sodium bisulfite was not added to the hydrolysates in this case. (See Section IV.) The hydrolysis was performed at 110° in a constant temperature, circulating oil bath for exactly 20 hours in each case. The hydrolysates were concentrated to dryness in vacuo over NaOH at room temperature. The residues were dissolved in 0.2 N sodium citrate buffer, pH 2.2, and analyses were performed on aliquots containing an equivalent of 0.2 to 0.4 μmole of glycopeptide. The results of the analyses on glycopeptides 2, 3 and 4 are recorded in Table 9. The stock solution of glycopeptide 5 was found by this procedure to contain 0.18 μmole of aspartic acid/ml. The concentration of glutamic acid was only 0.04 μmole/ml and that of phenylalanine only 0.009 μmole/ml. Since a number of other amino acids (Thr,Ser,Pro,Gly,Val,Ileu and Leu) were present in concentrations ranging from 0.04 to 0.06 μmole/ml, the glutamic acid and phenylalanine were probably present as contaminants.

(iii) Carbohydrate Composition. The component sugars of glycopeptides 2, 3 and 4 were identified by means of paper chromatography (Section III-B-1). Chromatography was performed in n-butanol-pyridine-water (6:4:3, v/v) with the use of the multiple development technique. Thiobarbituric acid reagent was used for the detection of sialic acid, and neutral sugars were detected with p-anisidine phthalate reagent. Aliquots of the stock solutions containing 0.3 to 0.5 μmole of glycopeptide were hydrolyzed in dilute sulfuric acid, and the hydrolysates were prepared for chromatography as described in Section V-A-3-c. Each of these glycopeptides was found to contain galactose, mannose and fucose in the approximate molar ratio of 1 to 2 to 1. The ratios were estimated from the chromatograms by the procedure described in Section III-B-2.

Glucosamine was detected in each of these glycopeptides in conjunction with the amino acid analyses. Sialic acid was detected in the glycopeptide 4 preparation, but could not be detected in glycopeptides 2 and 3. The limited amounts of glycopeptides 1 and 5 did not permit a qualitative analysis for sugars other than glucosamine, which, as mentioned previously (Section VI-A-3-b-(ii)), was detected in both of these glycopeptides.

The results of quantitative carbohydrate analyses on glycopeptides 2, 3 and 4 are tabulated in Table 9. Details of the analytical procedures are given below.

Hexose. Duplicate aliquots of the stock solutions containing 0.1 to 0.2 μmole of glycopeptide were assayed for hexose by the orcinol-sulfuric acid method (Section III-B-3-a). The total hexose contents of glycopeptides 2, 3 and 4, as determined by this method, are 1.39 ± 0.00, 0.70 ± 0.01 and 3.06 ± 0.06 μmoles of 1:1 galactose-mannose equivalents/ml of stock solution, respectively. When these values are corrected for the fucose content of the glycopeptides (below) and the ratio of galactose to mannose (above), as described in Section III-B-3-a, they become 1.08, 0.55 and 2.53 μmoles of hexose (galactose plus mannose) per ml, respectively. The stock solution of glycopeptide 5 contained 0.79 ± 0.00 μmole of 1:1 galactose-mannose equivalents/ml. Since it is not known whether this glycopeptide contained fucose, and since the types of hexose present are not known, this value for total hexose cannot be corrected.

Glucosamine. Glycopeptides 2, 3, 4 and 5 were analyzed for glucosamine in conjunction with the quantitative amino acid analyses of these glycopeptides (Section VI-A-3-b-(ii)). The values obtained are 0.92, 0.49, 1.62 and 0.34 μmole of glucosamine/ml of stock solution, respectively.

When these values are corrected for approximately 47% loss due to destruction (Section IV), they become 1.7, 0.9, 3.1 and 0.6 μmoles/ml.

Glycopeptides 2, 3 and 4 were also analyzed for glucosamine by the photometric method of Levvy and McAllan (Section III-B-3-b). The values obtained from duplicate samples containing 0.03 to 0.05 μmole of glycopeptide are 1.51 ± 0.01, 0.70 ± 0.01 and 2.66 ± 0.06 μmoles of glucosamine/ml of stock solution, respectively.

Fucose. Duplicate samples of glycopeptides 2, 3, and 4, containing 0.05 to 0.1 μmole of glycopeptide were analyzed for fucose by the cysteine-sulfuric acid method of Dische and Shettles (Section III-B-3-c). The values obtained are 0.36 ± 0.00, 0.17 ± 0.01 and 0.64 ± 0.01 μmole/ml of stock solution, respectively.

Sialic Acid. The sialic acid content of glycopeptide 4 was determined on duplicate aliquots of stock solution, containing about 0.07 μmole of glycopeptide, by the thiobarbituric acid assay procedure of Warren (Section III-B-3-d). The uncorrected value obtained is 0.17 ± 0.01 μmole/ml of stock solution. When corrected for the inhibition of color development by fucose as described in Section III-B-3-d, the value becomes 0.18 μmole/ml. Thus, it appears that the inhibition by fucose at the level of its concentration in this sample is not very significant.

(iv) Molecular Weight Determination. The molecular weight of glycopeptide 2 was estimated by the sedimentation-equilibrium method of Archibald (101), essentially according to the procedure of Schachman (102). The determination was performed on a 1% solution of the glycopeptide in 0.15 M NaCl at a speed of 59,780 revolutions/minute. The pH of this solution was 6.5. The molecular weight, calculated from two photographs taken

TABLE 9

COMPOSITIONS OF GLYCOPEPTIDES FROM RABBIT γ-GLOBULIN[a]

Residue	Glycopeptide 2		Glycopeptide 3		Glycopeptide 4	
	Calculated No. of Residues[b]	Estimated Whole No. of Residues	Calculated No. of Residues[b]	Estimated Whole No. of Residues	Calculated No. of Residues[b]	Estimated Whole No. of Residues
Hexose	2.9	3	2.7	3	3.7	4
Glucosamine[c]	4.0 (4.6)	4	3.3 (4.3)	4	3.9 (4.5)	4
Fucose	1.0	1	0.8	1	0.9	1
Sialic Acid	None	0	None	0	0.26	0
Aspartic Acid	1.03	1	1.00	1	1.28	1
Glutamic Acid	1.94	2	1.05	1	2.97	3
Phenylalanine[d]	0.97	1	0.91	1	1.01	1

[a] See text for partial compositions of glycopeptides 1 and 5.

[b] The number of residues was calculated as described in Section VI-A-3-b-(v).

[c] The glucosamine values in parentheses were obtained in conjunction with the amino acid analyses by the method of Moore, Spackman and Stein and are corrected for destruction. The other values were obtained by the method of Levvy and McAllan (Section IV-B-3-b-(iii)).

[d] In addition to these amino acids, glycopeptide 4 contained small amounts of Thr, Ser and Gly equivalent to 0.17, 0.19 and 0.21 residues, respectively. Other amino acids were absent from these glycopeptide preparations or present in amounts equivalent to less than 0.1 of a residue.

19 and 27 minutes after full speed was attained, is 2918 ± 0.

(v) Correlation of Analytical Data. The number of residues of each component in glycopeptides 2, 3 and 4 (Table 9) was estimated from the concentrations of these components in the stock solutions. Since the concentrations of aspartic acid, glutamic acid and phenylalanine in these glycopeptides are in the ratio of 1 to 2 to 1, 1 to 1 to 1 and 1 to 3 to 1, respectively, the weighted average of the concentrations of these amino acids in each glycopeptide was taken as the concentration of the glycopeptide in the stock solution, except in the case of glycopeptide 4, in which the value for aspartic acid was omitted from the average since it is somewhat higher than the others. Calculated in this way, the fucose content of each of these glycopeptides is equivalent to one residue. Since not more than one residue of fucose is present in the intact γ-globulin (Section VI-A-1-b), not more than one residue can be present in each glycopeptide. Calculated from the number of residues estimated in this way, the molecular weights of glycopeptides 2, 3 and 4 are 1984, 1854 and 2275, respectively, assuming glucosamine to be present as the N-acetyl derivative. Although the number of residues of glucosamine obtained by the photometric method of Levvy and McAllan is smaller in glycopeptide 3 than in glycopeptides 2 and 4, the values obtained in conjunction with the amino acid analyses indicate these three glycopeptides have the same glucosamine content.

(vi) Peptide Structure. Peptide sequences were determined by chemical and enzymatic degradation techniques.

Edman Degradation

Glycopeptide 3 was submitted to the Edman degradation according to the

procedure described by Acher et al. (110). The techniques employed in the present work have been outlined by Light and Smith (111). To 0.5 μmole of the glycopeptide dissolved in 0.1 ml of water in a 3 ml glass-stoppered centrifuge tube was added 0.1 ml of a 1% solution of phenylisothiocyanate followed by 0.02 ml of 25% trimethylamine. The reaction mixture was placed in a 40° water bath for 2 hours to permit the coupling reaction to take place. The volume of the reaction mixture was then increased to 0.4 ml by the addition of water. This solution was extracted 8 times with equal volumes of benzene to remove excess reagent and pyridine. Centrifugation was used where necessary to facilitate separation of the aqueous and organic phases. The sample was taken to dryness in vacuo over NaOH and paraffin. Cleavage and cyclization of the phenylisothiocyanate derivative to form the phenylthiohydantoin (PTH) derivative was accomplished by dissolving the residue in 0.1 ml of glacial acetic acid-concentrated HCl (5:1, v/v) and maintaining this solution at 40° in a water bath for one hour. The sample, after evaporation to dryness in vacuo over NaOH, was dissolved in 0.5 ml of 0.01 N HCl. The PTH-amino acid was extracted from this solution with water-saturated, peroxide-free ether (0.5 ml x 3). The PTH derivatives were chromatographed in solvent systems A and F (112,113) and detected on the chromatograms with iodine-azide reagent (equal volumes of 0.01 M iodine in 0.5 M potassium iodide and 0.5 M sodium azide). After each degradation step an aliquot containing approximately 0.1 μmole of the glycopeptide degradation product was removed from the aqueous phase, hydrolyzed in 6 N HCl for 20 hours at 105° in a sealed capillary tube and analyzed on an automatic amino acid analyzer. The remaining aqueous phase was submitted to the degradation procedure again.

The PTH derivative of glutamine was detected in the ether extract of the first degradation step by chromatography in both solvent systems. In the case of the chromatogram developed in solvent A, an additional spot which had the same R_f as the authentic PTH derivative of glutamic acid was detected. In the second step, these same components and the PTH derivative of phenylalanine were detected. The molar ratios of the component amino acids and glucosamine of glycopeptide 3 are Glu, 1.05; Phe, 0.91; Asp, 1.00; and glucosamine, 2.33. The molar ratios in the peptide remaining after the first degradation step were found to be Glu, 0.54; Phe, 0.66; Asp, 1.00; and glucosamine, 2.10; after the second step the ratios were Glu, 0.41; Phe, 0.65; Asp, 1.00; and glucosamine, 2.50. (The glucosamine values are not corrected for loss due to destruction.)

A 0.5 μmole aliquot of glycopeptide 2 was submitted to the Edman degradation procedure in the manner described above. No PTH amino acid derivatives could be detected in two steps. The molar ratios of the component amino acids and glucosamine in the glycopeptide after the second degradation step were Glu, 1.80; Phe, 0.99; Asp, 1.00; and glucosamine, 2.38. These values are in close agreement with the molar ratios in the intact glycopeptide, which are Glu, 1.90; Phe, 0.95; Asp, 1.00; and glucosamine, 2.36. (Again, the glucosamine values are not corrected for loss due to destruction.)

Enzymic Degradation

Leucine Aminopeptidase (LAP). Aliquots of the stock solutions of glycopeptides 2, 3 and 4 were incubated with LAP at 40° for 22 to 24 hours. The incubation mixtures were made up as follows:

Glycopeptide, 0.10 to 0.15 μmole
0.02 ml of 0.5 M Tris buffer, pH 8.5
0.01 ml of 0.025 M $MgCl_2$
0.07 mg of LAP (C_1 = 35)
Deionized-distilled water to 0.15 ml

An enzyme blank with the same constituents minus the glycopeptide was treated in the same manner. After incubation, the incubation mixture was divided into two equal portions, and these portions were submitted to electrophoresis-chromatography simultaneously under the conditions described in Section III-A-1. One of each pair of "fingerprints" was sprayed with ninhydrin reagent.

In this manner the LAP hydrolysate of glycopeptide 3 was found to contain glutamine, phenylalanine and a component, L-1, which could not be distinguished from the intact glycopeptide under these conditions. A faint trace of aspartic acid was detected, but it was present in much smaller amounts than the other components. Glucosamine was not detected. None of these components were detected on a "fingerprint" of an equal aliquot of the enzyme blank. To verify the identification of glutamine, the area corresponding to glutamine in the duplicate, unsprayed "fingerprint" was eluted with water, and the eluate was hydrolyzed with 6 N HCl in a sealed capillary tube for 20 hours at 105°. The hydrolysis product was shown to be glutamic acid by paper chromatography with authentic glutamic acid in propanol-pyrophosphate buffer (30:70, v/v) as previously described (Section III-A-1). In the same manner, the ninhydrin-positive components of the acid hydrolysate of component L-1 were found to be qualitatively the same as the intact glycopeptide. An aliquot of this hydrolysate was shown by quantitative analysis on the automatic amino acid analyzer to contain Glu,0.08; Phe, 0.08; Asp, 0.14; and glucosamine, 0.40 μmole. The

glucosamine value is not corrected for destruction. The molar ratios of these components (0.6, 0.6, 1.0 and 3.0, respectively) indicate, in agreement with the Edman degradation studies, that the aspartyl residue is carboxyl-terminal and that the carbohydrate moiety is bound to the aspartyl residue. The increase in the molar ratio of glucosamine to aspartic acid above that found in the intact glycopeptide (2.3), together with the detection of a small quantity of aspartic acid in the LAP hydrolysate, indicates aspartic acid was released from the glycopeptide by the action of LAP.

The LAP hydrolysate of glycopeptide 4 was found to contain glutamic acid, glutamine, phenylalanine and a much smaller amount of aspartic acid. Two additional ninhydrin-positive substances were detected. One of these, L-1, could not be distinguished from the intact glycopeptide under the conditions of electrophoresis and chromatography employed; the second, L-2, had the same low R_f as the intact glycopeptide, but behaved as a neutral substance in the pyridine-acetate buffer (pH 6.4). These substances and the component identified as glutamine were eluted from the unsprayed duplicate "fingerprint" and hydrolyzed, and the ninhydrin-positive components of the hydrolysates were identified by paper chromatography, as above. In this way the identification of glutamine was verified and the compositions of components L-1 and L-2 were found to be qualitatively the same as the intact glycopeptide, namely, glutamic acid, aspartic acid, phenylalanine, and glucosamine. This indicates that glutamic acid or, possibly, glutamine occupies the amino-terminus of the intact glycopeptide since a neutral species of glycopeptide, component L-2, the hydrolysate of which contained glutamic acid, aspartic acid, phenylalanine and glucosamine, resulted from the cleavage of glutamic acid from the negatively charged intact glycopeptide. Since there

are 3 residues of glutamic acid in acid hydrolysates of glycopeptide 4, this information does not distinguish between an amino-terminal glutamyl residue and an amino-terminal glutaminyl residue.

To study the LAP hydrolysate of glycopeptide 4 quantitatively, 0.2 μmole of the glycopeptide in 0.3 ml of water was incubated for 20 hours at 40° in the presence of 0.1 mg of LAP (C_1 = 35), 0.015 ml of 0.025 M $MgCl_2$ and 0.03 ml of 0.5 M Tris buffer, pH 8.5. After incubation, the hydrolysate was diluted to 1.0 ml with 0.2 N sodium citrate buffer, pH 2.2, and submitted for analysis on an automatic amino acid analyzer (Section III-A-2). This low pH would stop the enzyme action, and, because of the low pH of this solution, the sample was kept in the frozen state prior to analysis to reduce the possibility of hydrolysis of glycosidic bonds. The enzyme hydrolysate was found to contain Glu, 0.031; Glu-NH_2, 0.024; Phe, 0.011; and Asp, 0.005 μmole. No glucosamine was detected.

Glycopeptide 2 was not susceptible to hydrolysis by LAP. Only the intact glycopeptide was detected on a "fingerprint" of the digestion mixture.

Carboxypeptidase. Aliquots (approximately 0.1 μmole) of glycopeptides 2 and 4 were incubated with carboxypeptidase (C_1 = 9.1) in 0.1 ml of 0.05 M Tris buffer, pH 8.0, for 24 hours at 40°. The molar ratio of enzyme to substrate was approximately 1 to 50. In each case only the intact glycopeptides could be detected on a "fingerprint" of the entire digestion mixture. As found previously, glycopeptide 2 gave a very weak ninhydrin test and could be detected only after heating the "fingerprint" at 105°. The carboxypeptidase preparation used was three times recrystallized pancreatic carboxypeptidase (Worthington) which was washed with distilled water and treated with diisopropylfluorophosphate (DFP) in a 1% solution of LiCl, essentially as des-

cribed by Harris (97), and dialyzed against distilled water to remove excess DFP and other low molecular weight substances.

Chymotrypsin. A 0.1 μmole aliquot of glycopeptide 4 was incubated at 40° with chymotrypsin in 0.1 ml of 0.05 M Tris buffer, pH 8.0, for 22 hours. The molar ratio of enzyme to glycopeptide was about 1 to 50. The entire incubation mixture was submitted to electrophoresis-chromatography as described above. Only the intact glycopeptide could be detected with ninhydrin reagent.

Papain. Although papain will readily hydrolyze peptide bonds of neutral and basic amino acids in the pH range employed for the papain hydrolysis of rabbit γ-globulin (pH 6.3 to 6.8), it is unable to hydrolyze peptide bonds of glutamic acid under these conditions. At pH 4, however, where ionization of the γ-carboxyl group of glutamic acid is suppressed, papain is able to hydrolyze such peptide bonds (87). Hill *et al.* (114) took advantage of this property of papain in elucidating the amino acid sequence of an abnormal peptide from hemoglobin G. Since glycopeptide 4 contains glutamic acid, it seemed likely that it might be susceptible to hydrolysis by papain at or near pH 4. This was found to be the case, as indicated below.

Glycopeptide 4 was incubated with papain at pH 4.25 for 24 hours at 40°. The incubation mixture consisted of the following:

Glycopeptide 4, 0.5 μmole in 0.15 ml of water
0.015 ml of 0.2 M sodium acetate buffer, pH 4.25
0.075 ml of 0.1 M BAL (2,3-dimercaptopropanol)
0.030 ml of mercuripapain solution (5.7 mg/ml, C_1 = 1.5)

To avoid oxidation of BAL and subsequent polymerization of the oxidation products, which interferes with paper electrophoresis-chromatography, the incubation was performed under reduced pressure (approximately 15 mm of Hg)

in a small tube fitted with a stopcock. At the end of the digestion period, 0.005 ml of 1 M sodium bisulfite was added to the digestion mixture. An aliquot of the digest was submitted to paper electrophoresis-chromatography under the conditions described in Section III-A-1, and the electrophoresis-chromatogram was sprayed with ninhydrin reagent. This electrophoresis-chromatogram is reproduced in Figure 6.

The hydrolysis products, peptides P-1, P-2 and P-3, and the components identified in Figure 6 as glutamine and glutamic acid were isolated from the remainder of the digestion mixture by preparative paper electrophoresis and chromatography. Another component, designated GL-4, was identified as the intact glycopeptide 4 on the basis of its electrophoretic and chromatographic properties. The identification of glutamine and glutamic acid in the hydrolysate was confirmed by re-chromatography of these components with the authentic amino acids in n-butanol-acetic acid-water (200:30:75, v/v). In addition, the product of acid hydrolysis of the component identified as glutamine was found by paper chromatography in this solvent system to be glutamic acid. In the same manner, the acid hydrolysates of P-1 and P-2 were found to contain only glutamic acid. These two peptides were digested with LAP for 23 hours under the conditions described in Section VI-A-3-b-(vi). Electrophoresis-chromatography of the digestion mixtures showed that the peptides were completely hydrolyzed under these conditions and that the hydrolysis products were glutamic acid and glutamine in each case. As estimated from the electrophoresis-chromatograms, peptide P-1 contained one residue each of glutamic acid and glutamine, whereas P-2 contained one residue of glutamic acid and two residues of glutamine. These compositions are consonant with the

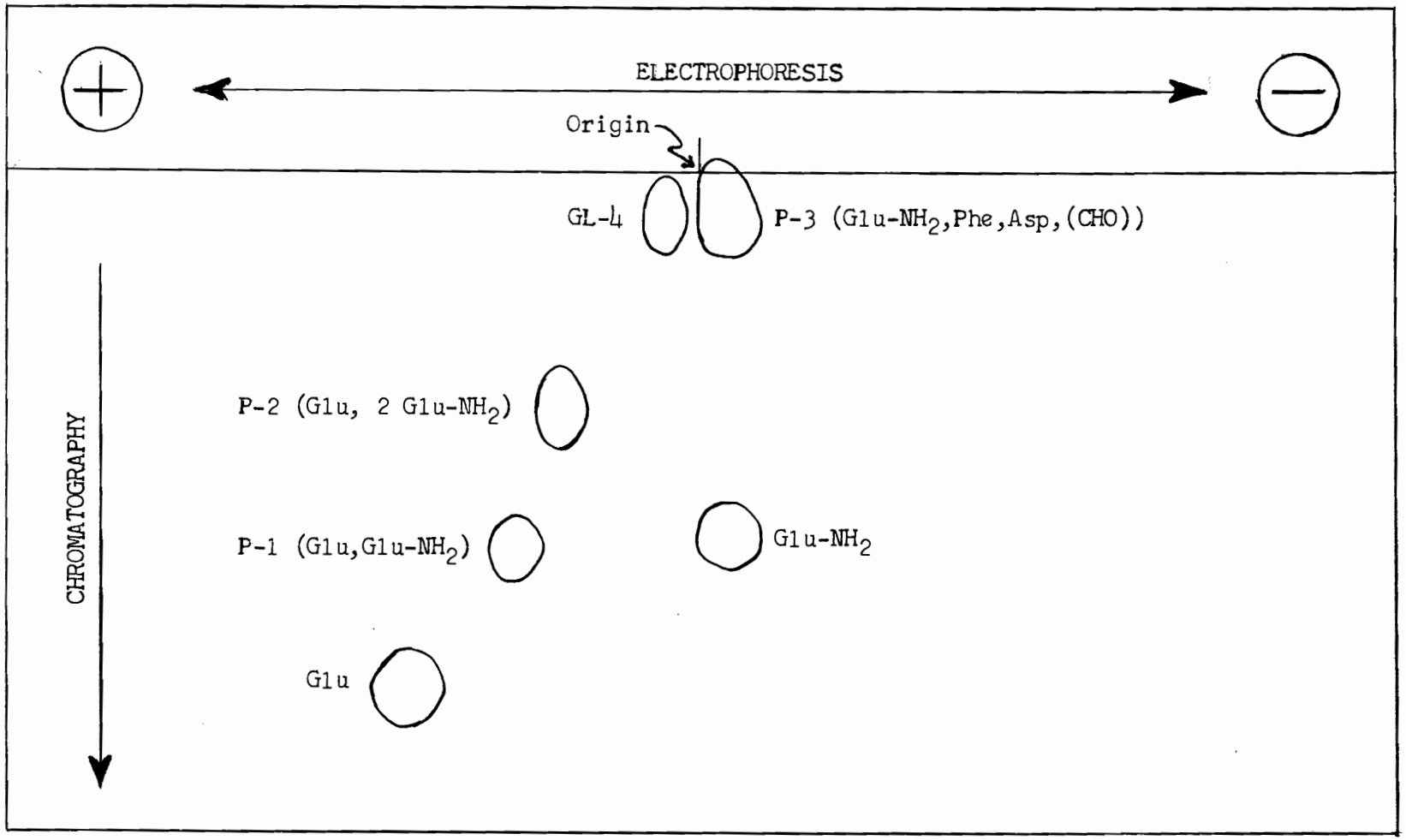

Figure 6. Electrophoresis-chromatogram of a papain hydrolysate of glycopeptide 4 (GL-4) from rabbit γ-globulin. Electrophoresis was performed in pH 6.4 pyridine-acetate buffer and chromatography in n-butanol-acetic acid-water (200:30:75, v/v). "CHO" is an abbreviation for carbohydrate.

electrophoretic and chromatographic properties of these peptides. An aliquot of an acid hydrolysate (6 N HCl, 105°, 20 hours) of the neutral component P-3 was shown by quantitative analysis on the automatic amino acid analyzer (Section III-A-2) to contain Glu, 0.08; Phe, 0.09; Asp, 0.11; and glucosamine, 0.328 μmole. (The glucosamine value is not corrected for destruction (Section IV).) Approximately 0.2 μmole of this neutral glycopeptide (P-3) was digested with LAP for 48 hours under the conditions previously described (Section VI-A-3-b-(vi)). Glutamine and phenylalanine were identified as the major amino acid components of the LAP hydrolysate by paper electrophoresis-chromatography of a portion (about 0.1 μmole) of the hydrolysate in the manner described in Section III-A-2; only a trace of aspartic acid was detected. A third major ninhydrin-positive component was indistinguishable from P-3 by its electrophoretic and chromatographic behavior under these conditions. This component was eluted from an electrophoresis-chromatogram of a second 0.1 μmole aliquot of the hydrolysate and hydrolyzed in 6 N HCl at 105° for 20 hours in a sealed capillary tube. The major ninhydrin-positive components of the acid hydrolysate were identified by paper electrophoresis-chromatography as glutamic acid, phenylalanine, aspartic acid and glucosamine. Of the amino acids, aspartic acid was present in the greatest quantity; phenylalanine and glutamic acid were present in approximately equimolar quantities.

B. Summary and Conclusions

1. Glycopeptides from Inert γ-Globulin and Antipneumococcal Antibody. A glycopeptide was isolated from the "inert" γ-globulin fraction and from the antibody fraction of a γ-globulin preparation from rabbit Type VII Pneumococcus antiserum. The glycopeptides were isolated from papain hydrolysates

of these fractions by the method developed by Rosevear and Smith (29) for isolation of glycopeptides from human γ-globulin. Further purification was accomplished by paper chromatography.

The composition of the glycopeptide isolated from the inert γ-globulin fraction is tabulated in Table 7. The orcinol-positive components of this glycopeptide accounted for only about 20% of the total orcinol-positive components of the inert γ-globulin preparation. The 4 hexose residues of the glycopeptide were estimated to be made up of 1 galactose and 3 mannose residues. The calculated number of residues of each component shows there is more glutamic acid than phenylalanine or aspartic acid. Since the level of contaminating amino acids is low in this preparation, it is likely that the lack of good stoichiometry between these components is due to the presence in the preparation of more than one glycopeptide species. This is consistent with the isolation from another preparation of rabbit γ-globulin of glycopeptides which differ only in the number of glutaminyl or glutamyl residues they contain (below). Note, however, that there is a good stoichiometric relationship between aspartic acid, fucose, glucosamine and hexose.

The molecular weight of this glycopeptide was estimated by the sedimentation-equilibrium method to be 3,150 ± 5.7%, while that calculated from the chemical composition, as given in Table 7, is 2423. Glucosamine was calculated as N-acetylglucosamine; the justification for this is discussed below. Since all carbohydrate components regularly identified with γ-globulin, with the exception of sialic acid, were found in this glycopeptide, it seems unlikely that any of its components would have gone undetected. It is possible that dimerization of the glycopeptide may have taken place

under the conditions of the molecular weight determination by sedimentation-equilibrium, which would account for the discrepancy in the molecular weights.

The glycopeptide isolated from Type VII Pneumococcus antibody was found to be contaminated with the specific antigen (Type VII Pneumococcus capsular polysaccharide) used to precipitate the antibody. Attempts to separate the glycopeptide and the antigen were unsuccessful. Chromatography in 80% ethanol afforded partial separation, although the peptide material streaked badly. Thus, the possibility that the glycopeptide was involved in the combining site between antibody and antigen seems unlikely. The fact that the antibody was heat-denatured before hydrolysis with papain also makes it unlikely that the difficulty encountered in separating the glycopeptide and antigen can be attributed to anything more than a similarity in properties.

Because of the presence of antigen in this glycopeptide preparation, quantitative analyses were not performed. However, an acid hydrolysate of this preparation was found by paper electrophoresis-chromatography to contain glutamic acid, aspartic acid and phenylalanine. These amino acids appeared to be present in approximately equimolar quantities, as in the case of the glycopeptide from the inert γ-globulin. In addition, this preparation was shown to contain all of the carbohydrate components found in the inert γ-globulin glycopeptide plus all of those reported by Barker (100) to be present in Type VII Pneumococcus capsular polysaccharide. This suggests that these two glycopeptides are very similar if not identical.

2. Glycopeptides from Rabbit γ-Globulin from Antipneumococcal Antiserum. Five glycopeptides were isolated from a papain hydrolysate of γ-

globulin from rabbit Type VII Pneumococcus antiserum. In this instance, the inert γ-globulin and specific antibody were not separated. The fractionation procedure employed and the recovery from each step is outlined in Table 8. Glycopeptides 1, 2 plus 3, 4 and 5 (Figure 3) account for approximately 3, 37, 40 and 8%, respectively, of the orcinol-positive components of the γ-globulin preparation, which is a total of 88% recovery. Glycopeptides 1 and 5 were further purified by paper electrophoresis-chromatography and paper electrophoresis, respectively.

Electrophoretic Properties and Purity. All five glycopeptides behaved as homogenous substances when submitted to paper electrophoresis-chromatography (pyridine-acetate buffer, pH 6.4; n-butanol-acetic acid-water (200:30:75, v/v)). The electrophoretic mobilities of glycopeptides 1, 2, 3, 4 and 5 indicate that they have net charges of +1, -1, 0, -1 and -2, respectively (Figure 5). Glycopeptide 4, however, was shown to be heterogeneous by chromatography on DEAE cellulose (Figure 3) and by gel filtration (Figure 4). As indicated by gel filtration studies, glycopeptide 4 was contaminated by a small quantity of a carbohydrate-containing substance of greater size than glycopeptide 4. Glycopeptides 2 and 3 behaved as homogeneous substances under the conditions of gel filtration. The purity of these two glycopeptides is also demonstrated by their compositions.

Composition and Molecular Weight. The compositions of glycopeptides 2, 3 and 4 are summarized in Table 9. The amino acid compositions of these three glycopeptides differ only in the number of glutamyl residues and are alike in that each has one residue each of phenylalanine and aspart-

ic acid. The carbohydrate compositions of these glycopeptide preparations differ only in that glycopeptide 4 has one more residue of hexose than the others and contains a small, unstoichiometric amount of sialic acid, whereas no sialic acid was detected in the other two. In view of the other similarities in composition, it is probable that the sialic acid, the extra hexose residue and, possibly, part of the aspartic acid, which is present in an amount equivalent to 1.28 residues, are part of the carbohydrate-containing contaminant in this preparation, which, as noted above, appears to be larger than glycopeptide 4. In each glycopeptide the hexose consists of galactose and mannose in the approximate molar ratio of 1 to 2.

The small quantities of glycopeptides 1 and 5 isolated did not permit complete analysis of these substances. Glycopeptide 1, which was orcinol-positive, was found by qualitative analysis to contain the following ninhydrin-positive components in the approximate molar ratios indicated: glucosamine, > 2; glutamic acid, 2; aspartic acid, phenylalanine, arginine, leucine and threonine, 1 each; lysine, 0-1, and serine and/or glycine, 1-2.

The molar ratios of components of glycopeptide 5 determined by quantitative analyses are: hexose (uncorrected), 4.4; glucosamine (uncorrected), 1.9; and aspartic acid, 1.0. When corrected for destruction, the molar ratio of glucosamine becomes about 4. Phenylalanine was not detected. A number of other amino acids, including glutamic acid, were present in amounts equivalent to 0.2 to 0.3 mole per mole of aspartic acid. These were probably due to contamination. These results indicate that the molar ratio of aspartic acid to glucosamine in this glycopeptide is about the same as in glycopeptides 2, 3 and 4. The hexose value is not corrected for differences in the color yields of the components with respect to the hexose

standard since, except for glucosamine, the component monosaccharides were not identified. The hexose content of this glycopeptide appears to be greater than that of glycopeptides 2 and 3, the uncorrected hexose contents of which are 3.65 and 3.33 residues, respectively.

Calculated from the chemical compositions, the molecular weights of glycopeptides 2, 3 and 4 are 1984, 1854, and 2275, respectively, assuming glucosamine to be present as the N-acetyl derivative. As estimated by the sedimentation-equilibrium method, the molecular weight of glycopeptide 2 is about 2900. This is in reasonable agreement with the molecular weight obtained by the same method for the glycopeptide from the inert γ-globulin preparation, above. In both cases, there is a considerable discrepancy between the molecular weight obtained by this method and that calculated from the chemical composition. A possible reason for this discrepancy is discussed above.

Peptide Structure. The amino acid sequence of glycopeptide 3 was elucidated by the Edman degradation method, and the identification of the PTH-amino acid cleaved from the glycopeptide in each step was confirmed by amino acid analysis of the remaining glycopeptide. The sequence obtained in this manner was Glu-(NH_2?).Phe.Asp. After two degradation steps, aspartic acid and glucosamine remained present in the same molar ratio as in the intact glycopeptide. This, alone, does not necessarily mean the carbohydrate moiety is linked to the aspartyl residue; however, partial degradation with leucine aminopeptidase, which hydrolyzed the glycopeptide very slowly, produced free phenylalanine and glutamine, while aspartic acid was detected in the hydrolysate in much smaller amounts. A neutral, carbohydrate-containing substance was isolated from the enzyme digest which

appeared to be a mixture of intact glycopeptide and glycopeptide degradation products. Quantitative amino acid analysis of an acid hydrolysate of this substance showed that it contained glutamic acid, phenylalanine, aspartic acid and glucosamine in the molar ratios 0.6, 0.6, 1.0 and 3.0, respectively. (The glucosamine value is not corrected for destruction.) This information establishes the peptide structure of glycopeptide 3 as:

$$Glu\text{-}NH_2.Phe.Asp.(Carbohydrate)$$

That the carbohydrate occurs as a single moiety attached to the aspartyl residue is supported by the nature of the tripeptide itself; since degradation with leucine aminopeptidase established the presence of a glutaminyl residue in this peptide, the carboxyl-terminal aspartyl residue is left as the only residue containing a functional group to which the carbohydrate could be linked.

The peptide structure of glycopeptide 4 (GL-4) was studied by enzymatic methods. The results of these studies are summarized in Table 10.

Hydrolysis of glycopeptide 4 with papain at pH 4.25 yielded free glutamine and glutamic acid, the peptides P-1 and P-2, and a glycopeptide, P-3, which was neutral at pH 6.4; the intact glycopeptide 4 was also present in the hydrolysate, as seen in Figure 6. The major amino acid components of a 48 hour leucine aminopeptidase hydrolysate of P-3 were identified as glutamine and phenylalanine; only a trace of aspartic acid was detected. Aspartic acid was found to be the major amino acid component of a mixture of glycopeptides isolated from this leucine aminopeptidase hydrolysate. Like P-3, the glycopeptides of this mixture were neutral at pH 6.4. Because of the specificity of leucine aminopeptidase for amino-terminal amino acids, it is concluded from these results that the carbohydrate moiety is

bound to the aspartyl residue and that the aspartyl residue is carboxyl-terminal in P-3.

Like glycopeptide 3, glycopeptide 4 was hydrolyzed only slowly by leucine aminopeptidase. The major hydrolysis products were identified as glutamic acid, glutamine, phenylalanine and two glycopeptide components. Aspartic acid was present in only trace amounts, and no glucosamine was detected. One of the glycopeptides could not be distinguished from the negatively charged intact glycopeptide by its electrophoretic mobility in pH 6.4 pyridine-acetate buffer or by its R_f in n-butanol-acetic acid-water (200:30:75, v/v). The amino acid composition of an acid hydrolysate of this component was qualitatively the same as the intact glycopeptide. The second glycopeptide was neutral at pH 6.4, and, like the first, the amino acid composition of an acid hydrolysate of this component was qualitatively the same as the intact glycopeptide, which indicates that this component was a mixture of glycopeptides produced by the action of leucine aminopeptidase on glycopeptide 4. These findings indicated that a glutamyl residue occupies a position at or near the amino-terminus of glycopeptide 4. The molar ratios of the amino acids released from glycopeptide 4 by partial hydrolysis with leucine aminopeptidase were found by quantitative analysis to be glutamic acid, 1.00; glutamine, 0.77; phenylalanine, 0.36; and aspartic acid, 0.16. Since the composition of peptide P-2 (Table 10) establishes the presence of only 1 glutamyl and 2 glutaminyl residues in this glycopeptide, this information establishes glutamic acid as the amino-terminal amino acid in the glycopeptide and, thus, in peptides P-1 and P-2 as well. It can also be concluded from these results that the aspartyl residue is

carboxyl-terminal in the glycopeptide. Since peptide P-2 accounts for all of the glutamine in glycopeptide 4, the presence of this amino acid in P-3 provides an over-lap between these two peptides. From this information it is concluded that the peptide structure of glycopeptide 4 is as written in Table 10, below.

TABLE 10

SEQUENCE INFORMATION FOR GLYCOPEPTIDE 4 FROM RABBIT γ-GLOBULIN

Enzyme	Substrate	Products
Leucine Aminopeptidase	GL-4	(Glu,1.00; Glu-NH_2,0.77; Phe,0.36; Asp,0.16
Papain	GL-4	
Peptide:		
P-1		(Glu,Glu-NH_2)
P-2		(Glu,Glu-NH_2,Glu-NH_2)
P-3		(Glu-NH_2,Phe,Asp,(CHO))
Leucine Aminopeptidase	Papain Peptide P-3	Glu-NH_2,Phe,Asp.(CHO)
Sequence:		Glu.Glu-NH_2.Glu-NH_2.Phe.Asp.(CHO)

Glycopeptide 4 was found to be inert to carboxypeptidase and chymotrypsin. Because carboxypeptidase is specific for carboxyl-terminal amino acids, failure of this enzyme to attack this glycopeptide indicates that the carboxyl-terminus is blocked and gives further support to the conclusion that the carbohydrate moiety is bound to the carboxyl-terminal aspartyl residue.

Glycopeptide 2 was found to be only weakly ninhydrin-positive and could not be degraded by the Edman technique, nor was it susceptible to hydrolysis by leucine aminopeptidase. In view of these findings and the presence of glutamic acid in acid hydrolysates of the glycopeptide, it

seems likely that the amino-terminus of the peptide is occupied by pyrollidone carboxylic acid (PCA), which is formed by the cyclization of glutamine, resulting in the loss of the free amino group. The cyclization of amino-terminal glutaminyl residues occurs under relatively mild conditions (115) and could easily have occurred under the conditions employed in the preparation of these glycopeptides. This glycopeptide, like glycopeptide 4, was not susceptible to hydrolysis by carboxypeptidase. From this information, together with the similarity in the compositions of glycopeptides 2, 3 and 4, it seems very likely that the peptide structure of glycopeptide 2, as isolated, is:

$$PCA.Glu\text{-}NH_2.Phe.Asp.(Carbohydrate).$$

The structure of the peptide as it occurs in the protein would be:

$$Glu\text{-}NH_2.Glu\text{-}NH_2.Phe.Asp.(Carbohydrate).$$

The peptide structures assigned to glycopeptides 2, 3 and 4 are in agreement with the net charge on these glycopeptides. The negative charge on glycopeptide 2 is accounted for in that cyclization of an amino-terminal glutaminyl residue to form pyrollidone carboxylic acid would result in loss of a positively charged amino group, leaving a carboxyl group of the aspartyl residue as the only charged group.

Thus, glycopeptides 2, 3 and 4 appear to have over-lapping amino acid sequences, and evidence indicates that in each case the carbohydrate is linked to an aspartyl residue. This indicates that these glycopeptides arose from the same structure in the protein. Since only a covalent bond would be expected to survive the conditions to which the glycopeptides were subjected during isolation and in characterization studies, linkage of the carbohydrate to the aspartyl residue would entail an amide bond involving

glucosamine or an ester linkage involving any one of the monosaccharide components. Since it has been shown by Fruton (116) that papain does not split the amide group of glutamine and both glutamine and asparagine have been isolated from papain hydrolysates of proteins and peptides (29,111), papain apparently attacks amide and ester bonds involving only the α-carboxyl groups of glutamic and aspartic acid. This suggests that it is the β-carboxyl group of the asparty1 residue in the glycopeptides that is involved in the linkage between the carbohydrate and peptide moieties since, excluding the possibility that the asparty1 residue is carboxyl-terminal in the protein, papain must have split the protein between the asparty1 residue and the residue carboxyl-terminal to it. The conclusion that the carbohydrate group is linked to the asparty1 residue is consonant with the isolation of a glycopeptide (glycopeptide 5) in which aspartic acid was the only amino acid present in appreciable amounts. However, as discussed below, it is possible that this glycopeptide arose from a different structure than the others.

Since the electrical charges of glycopeptides 2, 3 and 4 at pH 6.4 are accounted for by their amino acid compositions and glycopeptides which were neutral at this pH were isolated from leucine aminopeptidase hydrolysates of glycopeptides 3 and 4, the amino groups of the glucosamine residues must occur in these glycopeptides in a bound form. Since glucosamine generally occurs in biological materials as the N-acetyl derivative (79), it is likely that the glucosamine residues in these glycopeptides are also in this form. As mentioned above, the possibility that an amino group of a glucosamine residue is involved in the linkage between the peptide and carbohydrate moieties also exists.

3. The Carbohydrate Component of Rabbit γ-Globulin. Normal rabbit γ-globulin prepared by the chromatographic procedure of Sober et al. (12) was found to contain glucosamine, galactose, mannose, fucose and sialic acid. Of these, only sialic acid was not detected in the glycopeptide isolated from the "inert" rabbit γ-globulin fraction and in glycopeptides 2, 3 and 4, except for a small, unstoichiometric amount in the latter. As previously mentioned, sialic acid has been found to be linked to polysaccharides by relatively labile glycosidic bonds which might not be expected to survive the conditions under which the glycopeptides were prepared. Quantitative analysis of this γ-globulin preparation indicated the presence of 6 to 7 residues of hexose and 1 residue each of fucose and sialic acid (calculated on the basis of a molecular weight of 160,000 for γ-globulin). The molar ratio of galactose to mannose was approximately 1 to 2. According to Porter (38), rabbit γ-globulin contains 0.6% glucosamine, which is equivalent to about 6 residues. Although the fucose content of each of the glycopeptides (Table 9) accounts for all of the fucose in the γ-globulin, the results indicate that the intact γ-globulin contains more hexose and, possibly, more glucosamine than is accounted for in the glycopeptides. The data of Smith et al. (19), however, suggest that the hexose content of rabbit γ-globulin may not be as high as indicated by these results. By a photometric orcinol-sulfuric acid method similar to that used in the present studies, these investigators found the total hexose content of rabbit γ-globulin to be 0.93%, as glucose equivalents. When corrected for the presence of one residue of fucose and the color yield of galactose and mannose (in the ratio of 1 to 2) relative to glucose (Table 1), this value becomes 0.29% hexose (as galactose and mannose), which is equivalent to 3 residues.

This value is in excellent agreement with the hexose content of glycopeptides 2, 3 and 4. Porter (38), on the other hand, has reported the hexose content of rabbit γ-globulin as 0.5%, which is equivalent to 5 residues. In this instance, the hexose composition was measured by a photometric anthrone method with glucose as a standard and was not corrected for interference by fucose and the differences in the color yields of galactose and mannose relative to the glucose standard. With the anthrone reagent, fucose has about the same color yield as glucose (117), whereas both galactose and mannose are reported to have considerably lower color yields (118,119).

Because the data indicate that the carbohydrate compositions of each of the glycopeptides may not account for all of the carbohydrate of the intact glycoprotein, there exist the possibilities that either part of the monosaccharide components were cleaved from the glycopeptides during isolation, or that there is at least one other carbohydrate moiety at another locus in the γ-globulin molecule. The finding of more hexose and glucosamine in the inert γ-globulin glycopeptide than in the others lends support to the first possibility. Other evidence supports the latter possibility. As mentioned in Section I, Porter (38) has reported the presence of hexose and glucosamine in two of three polypeptide fragments isolated from papain hydrolysates of rabbit γ-globulin and antibodies. Evidence indicates that the three fragments arose from different parts of the protein molecule and, thus, that there are at least two carbohydrate moieties in the γ-globulin molecule. Proof that these fragments do not contain over-lapping sequences is not unequivocal, however. Calculated on the basis of the reported molecular weights of these fragments, the hexose and hexosamine content of the largest of the three fragments accounts for about 85% and that of the second

fragment for about 26% of the hexose and hexosamine content of rabbit γ-globulin, for a total recovery of 111%. The fucose and sialic acid contents were not reported.

As pointed out previously, the amino acid sequences and carbohydrate compositions of glycopeptides 2, 3 and 4, which, together, account for approximately 77% of the orcinol-positive components of rabbit γ-globulin, suggest that these glycopeptides arose from the same structure in the protein molecule. That this must be true is supported by the finding of one residue of fucose in each of these glycopeptides since analysis indicates there is no more than one residue of fucose in the intact glycoprotein. The partial compositions of glycopeptides 1 and 5, which account for an additional 11% (3% and 8%, respectively) of the total orcinol-positive components of the rabbit γ-globulin preparation, suggest that these substances could have arisen from this same structure, although the ratio of hexose to aspartic acid is somewhat higher in glycopeptide 5 than in glycopeptides 2 and 3.

The picture is complicated by the fact that the γ-globulin preparation from which the five glycopeptides were isolated contained approximately 6% α-globulin, which is known to contain more carbohydrate than γ-globulin (9). To explain the greater acidity of glycopeptide 5, it must be assumed that this glycopeptide either contains more than one aspartyl residue or that it contains sialic acid, or both. If it contains more than one aspartyl residue, the carbohydrate moiety would necessarily be considerably larger than that of glycopeptides 2, 3 and 4. In any event, glycopeptide 5 appears to have a different carbohydrate composition from the others, and it is possible that it arose from the α-globulin, the carbohydrate component of which is

otherwise unaccounted for. The small amount of contaminating glycopeptide in the glycopeptide 4 preparation, which, based on gel filtration properties, is larger than glycopeptide 4, may also have arisen from α-globulin. Since these glycopeptides were isolated in such small quantities on a molar basis, it seems unlikely that they arose from a second locus in the γ-globulin molecule. Moreover, if the carbohydrate of rabbit γ-globulin is distributed as indicated by Porter's studies, a glycopeptide arising from a second locus in the glycoprotein would be expected to contain less rather than more carbohydrate than glycopeptides 2, 3 and 4, since these glycopeptides account for approximately 77% of the orcinol-positive components of the intact γ-globulin.

Thus, although the possibility exists that rabbit γ-globulin contains more than one carbohydrate moiety, the evidence discussed above indicates that this glycoprotein, like human γ-globulin and egg albumin, contains only a single carbohydrate moiety which is bound by covalent linkage to an aspartyl residue of the protein.

VII. BOVINE IMMUNE LACTOGLOBULIN

A. Experimental Methods and Results

1. Carbohydrate Composition.

a. Qualitative Analysis. The component carbohydrates of bovine immune lactoglobulin were identified by paper chromatography as previously described (Section VI-A-1-a). The monosaccharide components were identified as glucosamine, galactose, mannose, fucose and sialic acid. The molar ratios of galactose, mannose and fucose, estimated from the chromatograms as described in Section III-B-2, are approximately 6, 3 and 2, respectively.

b. Quantitative Analysis. Quantitative carbohydrate analyses were performed on a stock solution of the immune lactoglobulin. The glycoprotein was dissolved in 0.9% NaCl, dialyzed against this solvent (20 volumes x 3) at 5° and filtered through a sintered glass funnel. The protein nitrogen concentration was determined as described in Section III-C, and the protein concentration (44.6 mg/ml) was calculated from the nitrogen concentration, based on a nitrogen content of 15.5% for this preparation (40).

Hexose. Duplicate 0.25 ml aliquots of stock solution (11.2 mg of protein) were analyzed for hexose by the photometric orcinol-sulfuric acid method (Section III-B-3-a). The total hexose value obtained is 1.09 ± 0.00%,

or 10.7 moles of 1:1 galactose-mannose equivalents per mole of protein. When this value is corrected for the fucose content (given below) and the ratio of galactose to mannose, as previously described (Section III-B-3-a), it becomes 0.93%, or 9.2 moles of hexose (as galactose and mannose) per mole of protein.

Fucose. The fucose content of the immune lactoglobulin, as determined on duplicate 0.20 ml aliquots of the stock solution (8.9 mg of protein) by the method of Dische and Shettles (Section III-B-3-c) is 0.18 ± 0.1%, which is equivalent to 2.0 moles of fucose per mole of protein.

Sialic Acid. Analysis of 0.1 ml aliquots of stock solution (4.5 mg of protein) by the thiobarbituric acid assay method (Section III-B-3-d) indicated that the immune lactoglobulin contains 0.29% sialic acid, which is equivalent to 1.5 moles of sialic acid (calculated as N-acetylneuraminic acid) per mole of protein. This value has been corrected for interference by fucose in the manner previously described. The uncorrected values obtained with duplicate samples agreed with this value within 2%, indicating again that interference by these relatively low levels of fucose is negligible.

2. Preparation of Glycopeptides. Thirty grams of heat-denatured immune lactoglobulin was digested with papain, and glycopeptides were isolated from the papain hydrolysate by the method described in Section VI-A-3 (Steps 1-5), with the exceptions noted below. The fractionation was followed by the photometric orcinol-sulfuric acid assay for hexose and by a photometric ninhydrin method, as before.

Hydrolysis of the immune lactoglobulin with papain (Step 1) proceeded

to apparent completion in 19 hours, as indicated by photometric ninhydrin assays. Only a small amount of insoluble material remained in the digestion mixture after this time; this was removed by centrifugation.

Although recovery of the orcinol-positive components from the Dowex 50 column (Step 2) was essentially quantitative, only approximately 41% of the ninhydrin-positive material was removed in this step, and, therefore, Steps 1 and 2 were repeated on the carbohydrate-rich material in the resin column eluate. In this instance, hydrolysis with papain was performed for 18 hours. The recovery of the orcinol-positive components from the resin column was again essentially quantitative, but little or no ninhydrin-positive material was removed by this procedure. This suggested that either the resin was not functioning properly or that the ninhydrin-positive material was in the form of large fragments which would not be bound by the resin.

Therefore, the resin column eluate, dissolved in 50 ml of water, was dialyzed against water (2 volumes x 6) at 5°. Approximately 75% of the orcinol-positive material remained in the dialysis tubing. The combined dialysates and the non-dialyzable fraction were lyophilized, and portions of the residues were further fractionated by gel filtration on a 3 cm x 160 cm column of Sephadex (Step 3). In this instance G-50 rather than G-25 Sephadex was employed. The columns were eluted with water at about 30 ml/hour and the effluents were collected in 8-10 ml fractions. The elution patterns of the orcinol-positive components of the dialyzable and non-dialyzable fractions were essentially the same. Thus, although these two fractions are referred to as "dialyzable" and "non-dialyzable," the same orcinol-positive components were probably present in both. Because

it contained most of the orcinol-positive components of the papain hydrolysate, only the non-dialyzable fraction was purified further. The Sephadex column elution diagram of this fraction is shown in Figure 8. Orcinol-sulfuric acid assays were run on 0.5 ml aliquots of every other fraction, and 0.1 ml aliquots of the same fractions were assayed by a photometric ninhydrin method. Since most of the ninhydrin-positive material emerged with the orcinol-positive components and desalting was achieved by dialysis, the remainder of the non-dialyzable fraction was further fractionated on DEAE cellulose (Step 4), without being submitted to the gel filtration step.

The non-dialyzable fraction was chromatographed in 1 g portions on a DEAE cellulose column (2.8 cm x 85 cm) in pH 8.4 Veronal buffer, as before. In this instance, however, the column was first eluted with 150 ml of 0.005 N Veronal buffer and then with a linear ionic gradient from 0.005 N Veronal to 0.05 M NaCl and 0.005 N Veronal over 3 liters of eluting buffer; from this point the column was eluted with the buffer of higher ionic strength. The effluent was collected in 20 ml fractions at the rate of about 60 ml/hour. An elution diagram is shown in Figure 7. Essentially all of the glycopeptide components emerged from the column in six major fractions, numbered 1 through 6 in order of their emergence.

Each of the fractions from Step 4 were fractionated and desalted on a 3 cm x 160 cm column of G-50 Sephadex (Step 5). None of the glycopeptides was obtained in pure form by this procedure. An elution diagram of fraction 2 is shown in Figure 8. Also shown in this figure is the elution diagram of the non-dialyzable fraction obtained in Step 3 with a G-50 Sephadex column of the same dimensions. A comparison of the elution

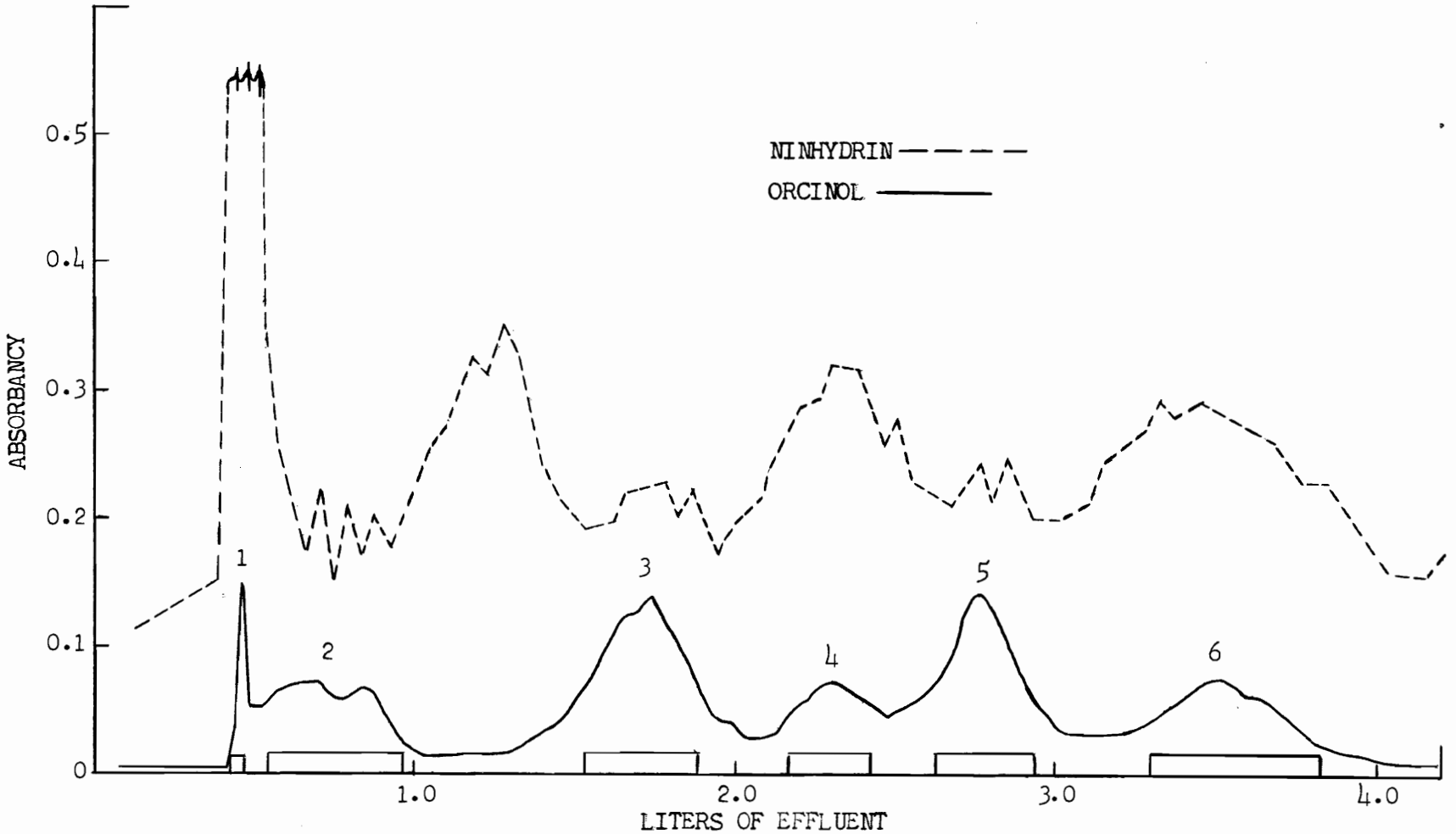

Figure 7. DEAE cellulose column elution diagram of glycopeptides from bovine immune lactoglobulin. The column was eluted with pH 8.4 Veronal buffer with an ionic gradient as described in the text. The blocks under each peak represent the fractions pooled.

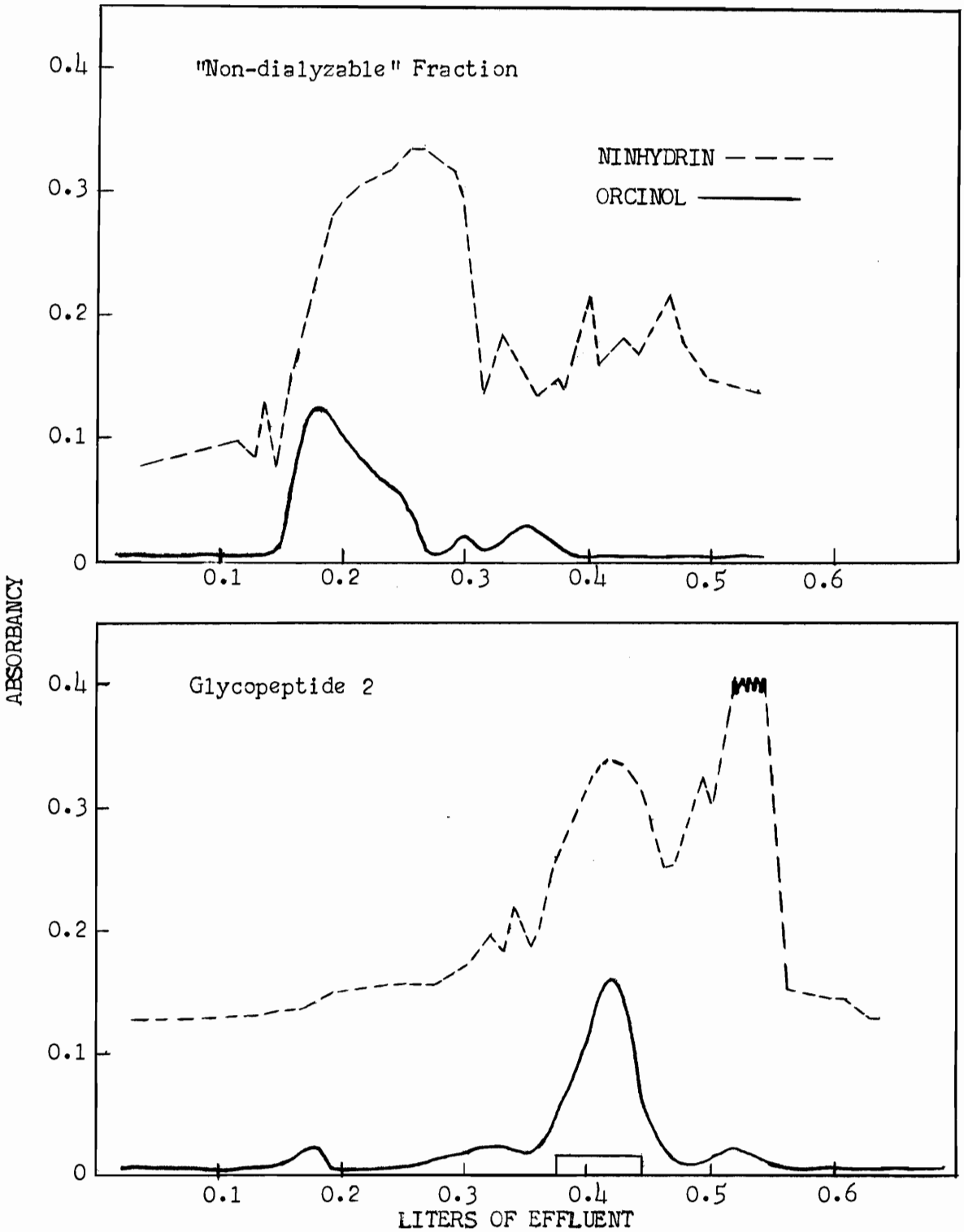

Figure 8. G-50 Sephadex column elution diagrams of "non-dialyzable" glycopeptide fraction and glycopeptide 2 from bovine immune lactoglobulin. A 3 cm x 160 cm column was used in each case, and the columns were eluted with water. The block under the glycopeptide 2 peak represents the fractions pooled.

patterns shows that glycopeptide 2 corresponds more closely to the last of the glycopeptide components of the non-dialyzable fraction to emerge from the column, which are presumably the smallest glycopeptides of the mixture. Glycopeptide fraction 2 was further fractionated on a G-25 Sephadex column (2.8 cm x 122 cm). The column effluent was collected in approximately 8 ml fractions, and orcinol and ninhydrin assays were run on 0.25 ml aliquots of every other fraction. An elution diagram appears in Figure 9.

The glycopeptide component of fraction 2 obtained from the G-25 Sephadex column behaved as a homogeneous substance when submitted to paper electrophoresis-chromatography under the conditions described in Section III-A-1. Attempts to obtain the other glycopeptides in pure form have been unsuccessful. However, the glycopeptide components of fractions 3, 5 and 6 were obtained in a form pure enough to permit semi-quantitative studies.

3. Characterization of Glycopeptides. For analysis, stock solutions of glycopeptide 2 and glycopeptide fractions 3, 5 and 6 were prepared in the manner described in Section VI-A-3-b. The concentration of glycopeptide 2 in the stock solution, based on the amino acid concentrations (below), was 0.87 μmole/ml.

a. Electrophoresis-Chromatography. Aliquots of glycopeptides 2 and fractions 3, 5 and 6 were submitted to paper electrophoresis-chromatography under the conditions described in Section III-A-1. The conditions were duplicated as nearly as possible in each case. The glycopeptides were detected on the electrophoresis-chromatograms with ninhydrin reagent. Glycopeptide 2 behaved as a neutral, homogeneous substance under these condi-

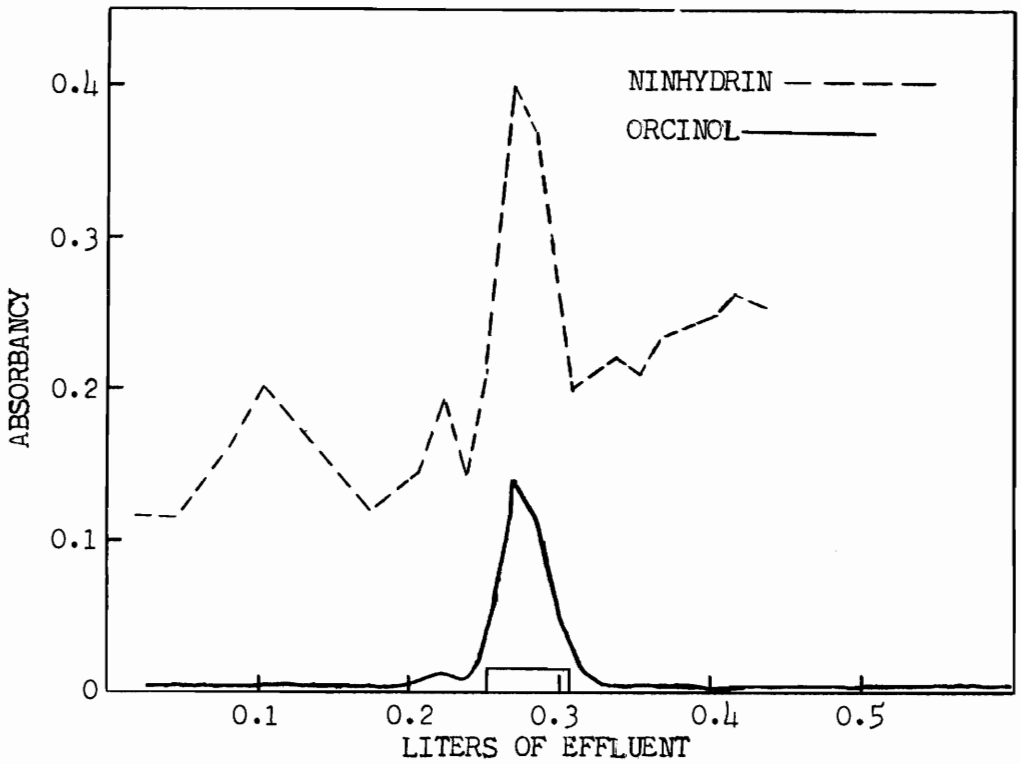

Figure 9. G-25 Sephadex column elution diagram of glycopeptide 2 from bovine immune lactoglobulin. The column was eluted with water. The block under the peak represents the fractions pooled.

tions and travelled only 1 cm from the origin in the chromatographic solvent. The spots representing glycopeptides 3, 5 and 6 were somewhat elongated in the direction of electrophoresis. These substances had approximately the same R_f as glycopeptide 2, but were acidic rather than neutral at pH 6.4. As expected from their behavior on DEAE cellulose (Figure 7), the order of electrophoretic mobility of these fractions was $6 > 5 > 3 > 2$.

b. Amino Acid Composition. The amino acid components of glycopeptides 2 and fractions 3, 5 and 6 were identified by paper electrophoresis-chromatography of acid hydrolysates of aliquots of the stock solution containing about 0.1 μmole of glycopeptide. Acid hydrolysis was performed in 6 N HCl at 105° for 20 hours. The major ninhydrin-positive components of

glycopeptide 2 and fraction 3 were identified as lysine, proline, arginine, glutamic acid, phenylalanine, aspartic acid and glucosamine. Glycopeptide fractions 5 and 6, the most acidic fractions, contained mainly glutamic acid, phenylalanine, aspartic acid and glucosamine. The glycopeptide 2 preparation contained only traces of other amino acids; the other preparations contained other amino acids in appreciable, but significantly smaller amounts.

Quantitative amino acid analyses were performed with an automatic amino acid analyzer (Section III-A-2). Aliquots of the stock solutions were hydrolyzed with 6 N HCl, and the hydrolysates were prepared for analysis as described in Section VI-A-3-b-(ii). Long and short column analyses were performed on equal aliquots of the hydrolysates containing an equivalent of 0.5 to 1 μmole of glycopeptide. The approximate molar ratios of the major amino acid components of glycopeptide fractions 3, 5 and 6 are listed in Table 11, below; the results for glycopeptide 2 are tabulated in Table 12.

TABLE 11

APPROXIMATE AMINO ACID COMPOSITIONS OF GLYCOPEPTIDE FRACTIONS 3, 5 AND 6 FROM BOVINE IMMUNE LACTOGLOBULIN

Residue	Fraction 3	Fraction 5	Fraction 6
Lysine	1	0.2	0.2
Proline	1	0.5	0.5
Arginine	1	0.1	0.2
Aspartic Acid	1-2	1-2	1-2
Glutamic Acid	3	3	3
Phenylalanine	1	1	1
Threonine	0.3	0.4	0.4
Serine	0.5	0.4	0.4

[a] Other amino acids were present in amounts smaller than the amount of threonine and serine in each fraction.

c. Carbohydrate Composition. The carbohydrate components of glycopeptide 2 were identified by paper chromatography as glucosamine, galactose, mannose and fucose; sialic acid was not detected. As estimated from the paper chromatograms, galactose, mannose and fucose were present in the approximate molar ratio of 2 to 3 to 1. The chromatographic procedures employed are described in Section VI-A-3-b-(iii). As previously mentioned, glucosamine was detected in glycopeptide fractions 3, 5 and 6 in conjunction with the amino acid analyses; qualitative and quantitative analyses for other sugars in these fractions were not performed.

The results of quantitative carbohydrate analyses on glycopeptide 2 are tabulated in Table 12. Details of the analytical procedures are given below.

Hexose. By analysis of duplicate aliquots containing about 0.1 μmole of glycopeptide by the orcinol-sulfuric acid method (Section III-B-3-a), the concentration of total hexose in the glycopeptide 2 stock solution was found to be 4.82 ± 0.00 μmoles of 1:1 galactose-mannose equivalents per ml. When corrected for the fucose content (below) and the ratio of galactose to mannose in the manner previously described, this value becomes 4.45 μmoles of hexose (as galactose and mannose) per ml.

Glucosamine. The glucosamine content of glycopeptide 2 was measured by two methods. Results of analysis of duplicate aliquots of the stock solution containing approximately 0.04 μmole of glycopeptide by the photometric method of Levvy and McAllan (Section III-B-3-b) indicated the presence of 3.04 ± 0.00 μmoles of glucosamine/ml. The uncorrected glucosamine value obtained in conjunction with the amino acid analysis (Section VII-A-b)

TABLE 12

COMPOSITION OF GLYCOPEPTIDE 2 FROM BOVINE IMMUNE LACTOGLOBULIN

Residue[a]	Calculated No. Of Residues[b]	Estimated No. Of Residues
Hexose	5.1	5
Glucosamine[c]	3.4 (4.1)	4
Fucose	0.9	1
Sialic Acid	None	0
Arginine	0.98	1
Lysine	0.96	1
Aspartic Acid	1.07	1
Glutamic Acid	2.90	3
Proline	1.01	1
Phenylalanine	1.01	1

[a] Ser, Gly and Thr were present in amounts equivalent to 0.19, 0.15 and 0.11 residue, respectively; other amino acids were absent or present in amounts equivalent to less than 0.1 residue.

[b] Calculated as described in Section VII-A-3-d.

[c] The glucosamine value in parentheses was determined in conjunction with the amino acid analysis by the method of Moore, Spackman and Stein and is corrected for loss due to destruction; the second value was obtained by the method of Levvy and McAllan (Section VII-A-3-c).

is 1.89 μmoles/ml of stock solution. When this value is corrected for approximately 47% loss due to destruction (Section IV), it becomes 3.6 μmoles of glucosamine/ml.

Fucose. Duplicate aliquots of stock solution containing about 0.1 μmole of glycopeptide 2 were assayed for fucose by the method of Dische and Shettles (Section III-B-3-c). The value obtained is 0.80 ± 0.02 μmole of fucose/ml.

d. Correlation of Analytical Data. The number of residues of each component in glycopeptide 2 (Table 12) was estimated from the concentrations of the amino acid components in the stock solution. Since the concentrations of arginine, lysine, aspartic acid, glutamic acid, proline and phenylalanine in the stock solution are in the ratio of 1:1:1:3:1:1, the weighted average of the concentrations of these amino acids (0.87 μmole/ml) was taken as the concentration of the glycopeptide in the stock solution.

e. Peptide Structure. The amino acid sequence of glycopeptide 2 was determined by enzymatic methods.

Leucine Aminopeptidase (LAP). An aliquot of the stock solution containing approximately 0.15 μmole of glycopeptide was digested with LAP at 40° for 50 hours. The incubation mixture was made up as previously described (Section VI-A-3-b-(vi)). By paper electrophoresis-chromatography under the conditions described in Section III-A-1, the amino acid components of the hydrolysate were identified as lysine, proline, arginine, glutamic acid, glutamine, phenylalanine and aspartic acid. Of these, lysine and glutamic acid were present in the largest amounts. No glucosamine was detected. In addition to the amino acids, three ninhydrin-

positive components of low R_f were present. One of these was identified as the intact glycopeptide 2 on the basis of its electrophoretic and chromatographic behavior. This substance was the major ninhydrin-positive component of the hydrolysate, indicating that LAP hydrolyzed glycopeptide 2 very slowly. The other two components had the same R_f as the intact glycopeptide but were negatively charged in pH 6.4 pyridine-acetate buffer. One of the two acidic components migrated about twice as far toward the positive pole as the other. The properties of these substances indicate that they were glycopeptides and suggest that the basic amino acids were cleaved from glycopeptide 2 before the acidic amino acids.

Papain Hydrolysis at pH 6.7. An aliquot of glycopeptide 2 was incubated with papain at 40° for 24 hours. The incubation mixture consisted of the following:

Glycopeptide 2, 0.15 μmole
0.10 ml of 0.1 M NaCN, pH 6.7
0.015 ml of mercuripapain solution (4.7 mg/ml, C_1 = 1.5)
Water to 0.25 ml

Only two basic peptides (P-1 and P-2) and an acidic glycopeptide were detected on a paper electrophoresis-chromatogram of an aliquot of the hydrolysate. Paper electrophoresis-chromatography was performed as described in Section III-A-1. Peptide P-1 and the glycopeptide were the major components and were present in approximately equimolar amounts; P-2 was present in much smaller amounts. These components were eluted from a second, unsprayed electrophoresis-chromatogram and hydrolyzed in 6 N HCl at 105° for 20 hours in sealed glass capillary tubes. The products of acid hydrolysis of P-1 were identified by paper electrophoresis-chromatography as lysine, proline and arginine in the approximate molar ratio of 1 to 1 to 1.

The acid hydrolysate of P-2 was found in the same way to contain lysine, proline, arginine and glutamic acid in approximately equimolar quantities. Since this peptide migrated only about half as far toward the negative pole as P-1, which contains two basic amino acid residues, the glutamic acid must have been present in P-2 as such rather than as glutamine. These two basic peptides are the same as two peptides, also designated "P-1" and "P-2," isolated from a pH 4.25 papain hydrolysate of glycopeptide 2 (below), an electrophoresis-chromatogram of which is reproduced in Figure 10. An aliquot of the acid hydrolysate of the acidic glycopeptide was found by analysis on an automatic amino acid analyzer (Section III-A-2) to contain glutamic acid, 0.147; phenylalanine, 0.053; aspartic acid, 0.054; and glucosamine, 0.149 μmole. The molar ratios of these components are 3, 1, 1 and 3, respectively. (The glucosamine value is not corrected for destruction.)

Papain Hydrolysis at pH 4.25. Glycopeptide 2 (0.5 μmole) was incubated with BAL-activated mercuripapain at 40° for 24 hours. The incubation was performed under reduced pressure to prevent oxidation of the BAL (2,3 dimercaptopropanol) and the incubation mixture was made up as described in Section VI-A-3-b-(vi). BAL rather than sodium cyanide was used to activate papain because of the volatility of the hydrogen cyanide formed at pH 4.25. An aliquot of the digestion mixture equivalent to about 0.1 μmole of glycopeptide was submitted to paper electrophoresis-chromatography under the conditions described in Section III-A-1, and the electrophoresis-chromatogram was sprayed with ninhydrin reagent. A reproduction of this electrophoresis-chromatogram is shown in Figure 10. Each of the components of the hydrolysate was isolated from the remainder of

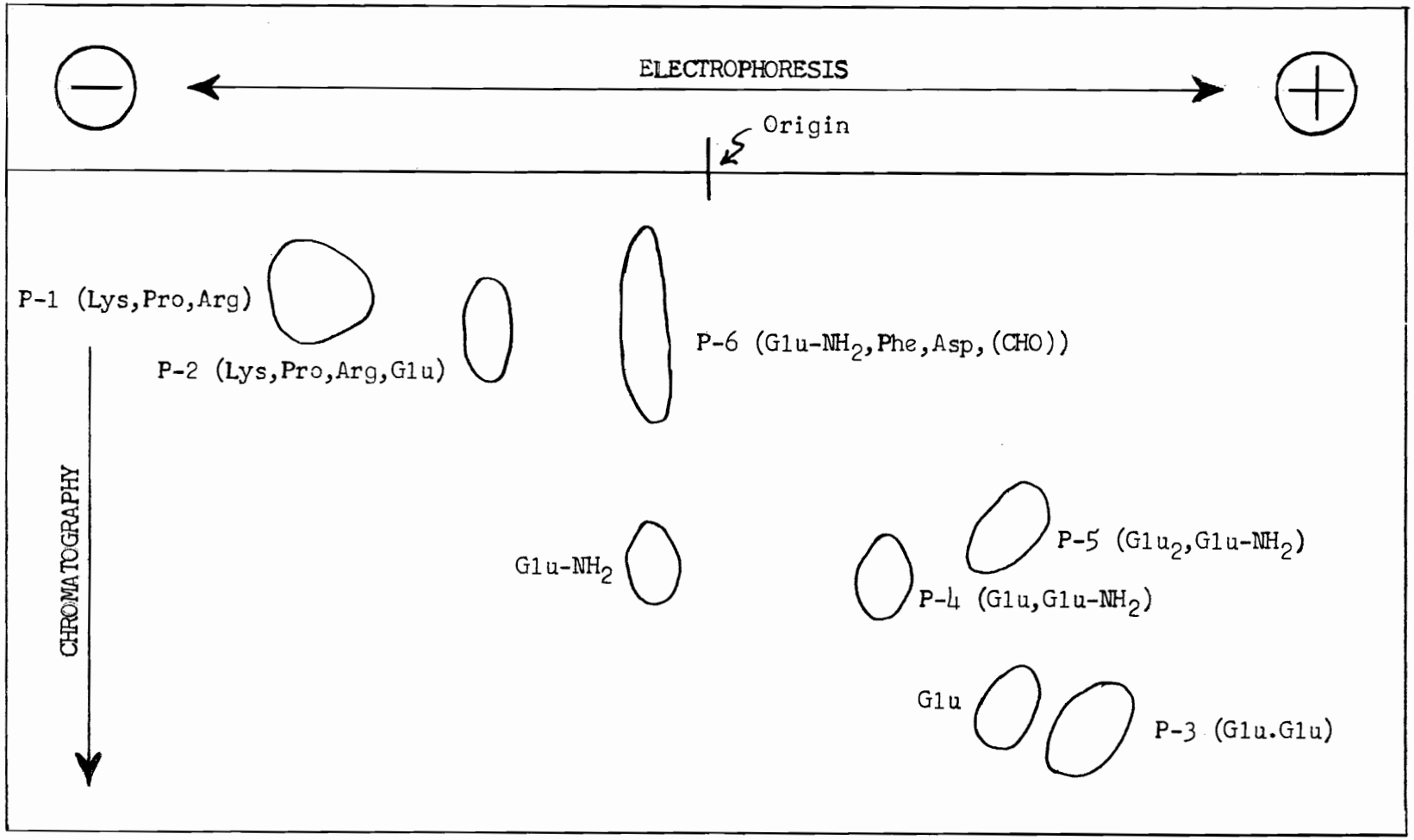

Figure 10. Electrophoresis-chromatogram of a papain hydrolysate of glycopeptide 2 from bovine immune lactoglobulin. Electrophoresis was performed in pH 6.4 pyridine-acetate buffer and chromatography in n-butanol-acetic acid-water (200:30:75, v/v). "CHO" is an abbreviation for carbohydrate.

the digestion mixture by preparative paper electrophoresis and paper chromatography in the same buffer and chromatographic systems.

The presence of glutamic acid and glutamine in the hydrolysate was confirmed by re-chromatography of these components with the authentic compounds in n-butanol-acetic acid-water (200:30:75). Furthermore, the product of acid hydrolysis of the component identified in Figure 10 as glutamine was shown to be glutamic acid by paper chromatography in the same solvent system.

Acid hydrolysis of peptides P-3, P-4 and P-5 yielded only glutamic acid, as identified by paper chromatography with authentic glutamic acid in the n-butanol-acetic acid-water system. These peptides were digested with LAP for 23 hours under the conditions described in Section VI-B-3-b-(vi). By paper electrophoresis-chromatography, the products of hydrolysis of P-4 and P-5 were identified as glutamine and glutamic acid; only glutamic acid was detected in the hydrolysate of P-3. Hydrolysis was complete in each case. The number of residues of glutamine and glutamic acid in P-4 and P-5, shown in Figure 10, was estimated from the paper electrophoresis-chromatograms. Since peptide P-5, which contains 2 glutamyl residues and 1 glutaminyl residue, accounts for all of the glutamyl and glutaminyl residues in glycopeptide 2, the presence of only glutamic acid in peptide P-3, together with the electrophoretic mobility of this peptide, establishes its sequence as glutamylglutamic acid.

An aliquot of P-6 was hydrolyzed in 6 N HCl at 105° for 20 hours in a sealed glass capillary tube. The hydrolysate was found by analysis on an automatic amino acid analyzer to contain glutamic acid, 0.019; phenylalanine, 0.055; aspartic acid, 0.062; and glucosamine, 0.166 μmole.

The molar ratios of these components are 0.3, 0.9, 1.0 and 2.7, respectively. (The glucosamine value is not corrected for destruction.) The presence of unstoichiometric amounts of glutamic acid in the hydrolysate indicates that this was a mixture of neutral glycopeptides. A second aliquot (approximately 0.2 μmole) of P-6 was incubated with LAP for 48 hours at 40°. The incubation mixture was made up as described in Section VI-A-3-b-(vi). The incubation mixture was divided into two equal aliquots, and the aliquots were submitted to paper electrophoresis-chromatography under the conditions described in Section III-A-1. By spraying one of the electrophoresis-chromatograms with ninhydrin reagent, the major ninhydrin-positive components of the hydrolysate were identified as glutamine, phenylalanine and a neutral glycopeptide component indistinguishable from P-6 under these conditions; aspartic acid was present in much smaller amounts. The neutral glycopeptide component was eluted from the second, unsprayed electrophoresis-chromatogram and hydrolyzed in 6 N HCl at 105° for 20 hours in a sealed glass capillary tube. The major ninhydrin-positive components of the acid hydrolysate were identified by paper electrophoresis-chromatography as aspartic acid and glucosamine; phenylalanine and glutamine were also present, but in significantly smaller amounts. This composition establishes the aspartyl residue as being the point of attachment between the peptide and carbohydrate moieties of the glycopeptide, and the specificity of leucine aminopeptidase places the glutaminyl and phenylalanyl residues amino-terminal to the aspartyl residue. The presence of less than one residue of glutamic acid in the acid hydrolysate of P-6 (above) indicates that the glutaminyl residue is amino-terminal in the tripeptide.

Prolidase. Prolidase is known to be specific for imido bonds of peptides of proline and hydroxyproline. Although previous evidence (47, 120) indicated that prolidase is specific for such imido bonds of dipeptides, recent studies by Hill and Schmidt (121) indicate that it will cleave the amino-terminal acid from larger peptides in which proline is the second residue from the amino-terminus.

The composition of peptide P-1 from the papain hydrolysate of glycopeptide 2 (Figure 10) shows that proline, lysine and arginine are in sequence in the glycopeptide, and the specificity of papain (86,87) suggested that either lysine or arginine was carboxyl-terminal in this tripeptide. The results of analysis of an LAP hydrolysate of glycopeptide 2 (above) indicated that lysine is amino-terminal in the glycopeptide. This information suggested that glycopeptide 2 has the amino-terminal tripeptide sequence Lys.Pro.Arg, and, thus, that it may be susceptible to hydrolysis by prolidase.

An aliquot of glycopeptide 2 was incubated with prolidase for 24 hours at 40°. The incubation mixture consisted of the following:

Glycopeptide 2, 0.15 μmole
0.02 ml of 0.5 M Tris buffer, pH 8.0
0.01 ml of 0.025 M $MnCl_2$
0.01 ml of prolidase solution (8 mg/ml, C_1 = 2)
Water to 0.20 ml

The purified prolidase preparation (Section II) was dialyzed against 0.005 M Tris buffer, pH 8.0 (100 volumes x 1), at 5° just before use. An aliquot of the digestion mixture equivalent to about 0.1 μmole of glycopeptide was submitted to electrophoresis-chromatography as described in Section III-A-1. The electrophoresis-chromatogram was sprayed with ninhydrin and then with a modified Sakaguchi reagent (122) for the detection of arginine.

The components of the digestion mixture were identified as lysine and two arginine-containing components, Pr-1 and Pr-2. No other components were detected by this means. Pr-1 was the major component and was identified as the intact glycopeptide 2 on the basis of its electrophoretic and chromatographic properties under these conditions. Pr-2 had the same low R_f as glycopeptide 2, but, unlike glycopeptide 2, which is neutral in the pH 6.4 pyridine-acetate buffer, it was acidic at this pH. This evidence indicates that Pr-2 was a glycopeptide which was produced by the cleavage of lysine from the amino-terminus of glycopeptide 2. This information establishes the sequence of peptide P-1 as Lys.Pro.Arg.

B. Summary and Conclusions

A glycopeptide has been isolated in a highly purified form from a papain hydrolysate of bovine immune lactoglobulin. The method of isolation was essentially the same as that outlined in Table 8. This glycopeptide (glycopeptide 2) behaved as a homogeneous substance under the conditions of gel filtration on G-25 Sephadex (Figure 9) and when submitted to paper electrophoresis-chromatography; its purity is further demonstrated by its composition, given in Table 12. Other glycopeptides (glycopeptide fractions 3, 5 and 6) were isolated from the hydrolysate in a less pure form; each of these was orcinol-positive and was found to contain glucosamine. Because of the lack of purity no further carbohydrate analyses have been attempted on these fractions. The approximate amino acid compositions of glycopeptide fractions 3, 5 and 6 have been estimated by quantitative analysis (Table 11); the results show that the amino acid compositions of these fractions are very similar to the amino acid composition of glycopeptide 2. The order of electronegativity

of these substances at pH 6.4 is 6 > 5 > 3 > 2.

The carbohydrate components of bovine immune lactoglobulin were identified as glucosamine, galactose, mannose, fucose and sialic acid. Quantitative analyses indicate that there are about 9 residues of hexose, 2 of fucose and 1 to 2 of sialic acid per molecule of protein, based on a molecular weight of 160,000 (40) for the glycoprotein. Galactose and mannose are in the approximate molar ratio of 6 to 3, as estimated from paper chromatograms. The same preparation of immune lactoglobulin has been reported by Smith et al. (84) to contain 1.5% hexosamine (as glucosamine), which is equivalent to about 15 residues of glucosamine.

The carbohydrate composition of glycopeptide 2 (Table 12) clearly does not account for all of the carbohydrate in the intact glycoprotein; the glycopeptide contains fewer residues of hexose, glucosamine and fucose and no sialic acid. The estimated ratio of galactose to mannose (2 to 3) in glycopeptide 2 indicates that it contains all of the mannose of the intact immune lactoglobulin. A comparison of the gel filtration properties of glycopeptide 2 with those of a mixture of all the glycopeptides isolated from the papain hydrolysate of immune lactoglobulin (Figure 8) indicates that most of the glycopeptides were considerably larger than glycopeptide 2, and the similarities in the amino acid compositions of the various glycopeptide fractions (Tables 11 and 12) suggest that they may have come from the same amino acid sequence in the glycoprotein. The greater acidity of glycopeptides 5 and 6 is consonant with the absence from these glycopeptides of lysine, proline and arginine, which occupy the amino-terminal position in glycopeptide 2 (below). These considerations suggest that the carbohydrate moiety of glycopeptide 2 may

have arisen from a larger structure, part of which was lost during isolation.

The results of amino acid sequence studies on glycopeptide 2 are summarized in Table 13. The evidence shows that the carbohydrate moiety of the glycopeptide is attached to the peptide moiety by way of the aspartyl residue and establishes the amino acid sequence shown in the table.

TABLE 13

SEQUENCE INFORMATION FOR GLYCOPEPTIDE 2
FROM BOVINE IMMUNE LACTOGLOBULIN

Enzyme	Substrate	Products
Prolidase	GL-2	Lys + Acidic Glycopeptide
Papain	GL-2	
Peptide:		
P-1		(Lys,Pro,Arg)
P-2		(Lys,Pro,Arg,Glu)
P-3		Glu.Glu
P-4		(Glu,Glu-NH_2)
P-5		(Glu,Glu,Glu-NH_2)
P-6		Glu-NH_2.(Phe,Asp,(CHO))
Leucine Aminopeptidase	Papain Peptide P-6	Glu-NH_2, Phe,Asp.(CHO)
Sequence:		Lys.Pro.Arg.Glu.Glu.Glu-NH_2.Phe.Asp.(CHO)

Inasmuch as both glycopeptide 2 and a glycopeptide isolated from a papain hydrolysate of glycopeptide 2 (P-6, Table 13) were neutral at pH 6.4 and their electrophoretic properties can be accounted for by their amino acid compositions alone, the amino groups of the glucosamine residues in these glycopeptides must have been in a bound form. As suggested in the case of rabbit γ-globulin, it is likely that the glucosamine occurs as the N-acetyl derivative.

As mentioned previously, only covalent bonds would be expected to sur-

vive the conditions to which glycopeptide 2 was subjected. Thus, although further work will be required to determine the exact nature and number of carbohydrate moieties in bovine immune lactoglobulin, it can be concluded that at least part of the carbohydrate components of this glycoprotein are bound to the protein by a covalent bond involving an aspartyl residue.

VIII. DISCUSSION

The results of studies presented in this thesis can be summarized briefly as follows: (1) The carbohydrate moieties of glycopeptides isolated from rabbit γ-globulin and a glycopeptide from bovine immune lactoglobulin were shown to be bound to the peptide moieties by a covalent bond involving an asparty1 residue. (2) Evidence was presented which indicates that three glycopeptides isolated from human II-3 γ-globulin were the same as three isolated from the II-1,2 fraction of human γ-globulin by Rosevear and Smith (29). (3) The amino acid sequences and carbohydrate compositions of the glycopeptides from the three species were found to be very similar, and (4) the amino acid and carbohydrate compositions of three glycopeptides obtained in high yield from rabbit γ-globulin support the conclusion that they arose from the same locus in the γ-globulin molecule.

The amino acid sequences and carbohydrate compositions of the glycopeptides from the three species are summarized below.

Human*	Glu.Glu.Asp(NH_2).Tyr.Glu.Asp.	(8 glucosamine, 3 galactose, 5 mannose, 2 fucose, 1 sialic acid)
Rabbit	Glu.Glu(NH_2).Glu(NH_2).Phe.Asp.	(4 glucosamine, 1 galactose, 2 mannose, 1 fucose)
Bovine	Lys.Pro.Arg.Glu.Glu.Glu(NH_2).Phe.Asp.	(4 glucosamine, 2 galactose, 3 mannose, 1 fucose)

These glycopeptides illustrate some interesting differences and simi-

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* From the data of Rosevear and Smith (29).

larities among the species. Except for the occurrence of sialic acid in the human glycopeptide, the carbohydrate compositions are qualitatively alike but differ in the number of residues of each component. All three of the intact glycoproteins contain sialic acid, and, as previously mentioned, it is likely that sialic acid was lost from the rabbit and bovine glycopeptides during isolation. Analysis has shown that the carbohydrate compositions of human γ-globulin and bovine immune lactoglobulin are very similar with respect to their glucosamine, fucose, sialic acid and total hexose compositions, but differ in the relative amounts of galactose and mannose they contain; of the 8 hexose residues found in human γ-globulin, approximately 3 are galactose and 5 are mannose, whereas of the 9 residues found in the bovine glycoprotein approximately 6 are galactose and only 3 are mannose residues. Rabbit γ-globulin contains considerably less carbohydrate than these two glycoproteins.

The similarities and differences in the amino acid sequences of the three glycopeptides are emphasized by the manner in which they are arranged, above. The bovine glycopeptide contains an amino acid sequence almost identical to that found in the rabbit glycopeptide; the sequences differ only in that a glutamyl residue occurs in the bovine glycopeptide in place of a glutaminyl residue in the rabbit. A difference of this type, of course, may be artifactual. Although the glycopeptide from human γ-globulin is very similar to these, it can be seen that it differs in having a tyrosyl residue in place of the phenylalanine residues and an asparaginyl residue in place of the glutaminyl residues common to the other two species, and, in addition, the human glycopeptide contains an extra glutamyl residue between the aromatic amino acid residue and the

terminal aspartyl residue.

Recent studies on glycopeptides from human γ-globulin by Rothfus (123) have shown that a glucosamine residue in the carbohydrate moiety is attached to the aspartyl residue and has provided evidence that the carbohydrate-peptide linkage is an amide bond between the β-carboxyl group of the aspartyl residue and the amino group of glucosamine. According to a recent brief report by Winzler and Inouye (124), an analogous situation obtains in a glycopeptide from orosomucoid. These investigators have obtained evidence for an amide bond between the amino group of a glucosamine residue and the γ-carboxyl group of a glutamyl residue in the glycopeptide. As previously indicated, the possible involvement of the β-carboxyl group in the carbohydrate-peptide linkage in glycopeptides from human γ-globulin was suggested earlier by Rosevear and Smith (29) on the basis of the specificity of papain for α-carboxyl-linked peptide (or ester) bonds. The same argument can be advanced in favor of a β-carboxyl linkage in the glycopeptides from rabbit γ-globulin and bovine immune lactoglobulin, and, by further analogy with human γ-globulin, it might be predicted that the aspartyl residue in these glycopeptides is also linked to glucosamine by an amide bond. It should be mentioned in this connection that recent studies by Neuberger and coworkers (125) on a glycopeptide from egg albumin have led these investigators to the tentative proposal that the carbohydrate-peptide linkage in this glycopeptide involves an aspartylglycosylamine structure.

The observation that leucine aminopeptidase released free aspartic acid from the rabbit and bovine glycopeptides at a low but detectable rate is in apparent contradiction to the suggestion that the carbohydrate moieties of the glycopeptides are bound to the aspartyl residues through the β-carboxyl

group since this enzyme is also reported to be specific for α-carboxyl-linked bonds (either amide or ester) (126). A possible explanation for the susceptibility of the peptide-carbohydrate bonds to the action of leucine aminopeptidase which is compatible with the existence of a β-carboxyl linkage is afforded by the studies of Swallow and Abraham (127) and others (128,129), who have demonstrated that peptide bonds of aspartic acid may be interconverted to the α- or β-asparty1 compounds with the formation of an intermediate cyclic imide by the condensation of the free carboxyl group of the aspartic acid with the amide nitrogen. Such interconversions have been demonstrated with model peptides in acid solution, and the conversion of α-asparty1 peptides to β-asparty1 peptides in neutral, aqueous solution at 100° has also been demonstrated (128,129). Although the conversion of β-asparty1 to α-asparty1 peptides in neutral solution was not reported, it is possible that under the conditions of hydrolysis of the glycopeptides with leucine aminopeptidase enough of the α-linkage was formed to permit slow hydrolysis of the bond by this enzyme. Such a mechanism, of course, invokes an amide rather than an ester bond between the carbohydrate and peptide moieties.

In addition to carbohydrates, other non-amino acid constituents of proteins have been shown to be bound to the proteins by covalent bonds. The heme of cytochrome c is known to be bound to the protein portion through thioether bonds between cysteinyl residues and the vinyl side chains of the heme (130). It has also been demonstrated that certain coenzymes are bound to their apoenzymes by covalent linkage, and such bonds have been implicated in still other coenzyme-apoenzyme complexes. Lipoic acid has been demonstrated to be bound to the pyruvate and α-ketoglutarate de-

hydrogenase apoenzymes by an amide bond involving the carboxyl group of lipoic acid and an ϵ-amino group of a lysyl residue (131), and a structurally related coenzyme, biotin, appears to be bound to protein in the same manner. Biotin is known to occur associated with proteins, and biocytin (ϵ-N-biotinyl-L-lysine) has been isolated from yeast autolysate (132). Fischer et al. (133) have found that pyridoxal phosphate is bound to muscle phosphorylase through an ϵ-amino group of a lysyl residue and the aldehyde group of pyridoxal phosphate.

It should be pointed out that the isolation of glycopeptides from two different fractions of human γ-globulin, namely, the II-3 and II-1,2 fractions, which appear to be the same and arise from the same structure in the glycoprotein molecule and the isolation of glycopeptides in high yield from rabbit γ-globulin which also appear to arise from the same structure in the molecule reveals a high degree of chemical homogeneity in these γ-globulins, at least in the portion of the molecules from which the glycopeptides arose. Although studies on glycopeptides from bovine immune lactoglobulin were less complete, preliminary evidence suggests chemical homogeneity in this protein fraction as well. These findings are of particular interest in view of the physical heterogeneity of γ-globulins and the question of the nature of differences in γ-globulin fractions separable by physical means and the differences between antibody and "inert" γ-globulin, previously discussed (Section I).

Although γ-globulins have been studied extensively, the physiological significance of the carbohydrate portion of these glycoproteins remains to be determined. No evidence has been obtained to indicate that the carbohydrate components participate in the immunological reactions of γ-globulins. However, some inferences can be drawn from the information available.

Antibodies are now generally considered to be bivalent (1); that is, evidence indicates that the antibody molecule contains two antigen-binding sites. If, as indicated by the present studies and the work of Rosevear and Smith (29), human γ-globulin contains only one carbohydrate moiety, the carbohydrate cannot be a part of both binding sites, if, indeed, it participates in the antigen-antibody reaction at all. As previously mentioned (Section I), only one of two monovalent polypeptide fragments (fractions I and II) obtained from papain hydrolysates of rabbit antibodies by Porter (38) appeared to contain carbohydrate. This, again, indicates that the carbohydrate need not be a part of the antigen-binding sites of antibodies. The third and largest of the three polypeptide fragments (fraction III) obtained from the papain hydrolysates of the antibodies lacked the ability to combine with antigen but brought about the precipitation of most of the antibody to rabbit γ-globulin from goat and rat. Fractions I and II, on the other hand, contributed little to the antigenicity of the intact γ-globulin molecule. Since fraction III contained most of the carbohydrate of the intact γ-globulin molecule, these considerations leave open the possibility that the carbohydrate component may contribute significantly to the antigenicity of γ-globulin.

More recently, Nisonoff *et al.* (134) have made a significant contribution toward the understanding of the gross structure of γ-globulin. These workers found that fragments which appear to be the same as those produced by hydrolysis with papain could be obtained by hydrolysis of rabbit antibody with pepsin in the presence of cysteine or other disulfide-splitting agents. Peptic hydrolysis of 7 S γ-globulin in the absence of such agents produced

a fragment with a sedimentation constant of about 5 S which retained most of the capacity of the antibody to precipitate antigen, and, thus, was taken to be bivalent. This 5 S fragment could then be split by disulfide-splitting agents into fragments with sedimentation coefficients of about 3.5 S which exhibited the properties of monovalent fragments. Since papain is always activated with such disulfide-cleaving agents, it was suggested that the papain hydrolysis of γ-globulin proceeds by the same mechanism. Putnam and coworkers (135, 136) have studied the hydrolysis of γ-globulin from rabbit and other mammalian species by papain and have provided evidence that hydrolysis of a single bond highly susceptible to proteolysis results in formation of the fragments. Thus, it now appears that the two monovalent fragments are bound by disulfide bonds and that the antigenic fragment (fraction III) is separated from these fragments by hydrolysis of the susceptible bond. A study of the influence of the carbohydrate component on the antigenic properties of fraction III may prove to be profitable.

There is good evidence that the serological specificity of blood group substances are conferred upon them by their carbohydrate moieties (25,26). The blood group substances (also called isoagglutinogens) occur with other mucosubstances on the surface of erythrocytes. The isoagglutinogens will bind specific proteins (isoagglutinins) present in the serum of individuals of a different blood group. The agglutinin molecule binds more than one erythrocyte, giving rise to agglutination of the erythrocytes. Mucosubstances with blood group activity have also been found in various tissues, including the lung, liver, kidney, bone and muscle and in various mucinous secretions, such as saliva and gastric

juice. All of the blood group substances, regardless of source, have very similar gross analytical compositions (26). All contain four monosaccharide components: glucosamine, galactosamine, galactose and fucose. Sialic acid appears to be absent. The ratios of the component sugars vary from group to group. The ratio of the carbohydrate to polypeptide is high, the amino acid composition accounting for not more than about 25% of the total weight. Inactivation of the specific blood group substances has been achieved by partial hydrolysis with enzyme preparations from microorganisms which attack the polysaccharide portions of the mucosubstances. Proteolytic enzymes do not appear to destroy the activity (25,26). An enzyme preparation from Clostridium tertium is reported to destroy the A group substance in human saliva but not the B substance from the same source. A Bacillus fullminous enzyme preparation destroys blood group O substance but does not attack A and B substances. Loss of most of the fucose occurs simultaneously with loss of activity, and the ratio of glucosamine to galactosamine is decreased. The enzyme action is inhibited by fucose. This microorganism also contains enzymes which destroy A and B substances. The destruction of A substance was inhibited by acetylgalactosamine and that of B substance by galactose, and it has been suggested that these sugars are responsible for the specificity of the blood group substances (25).

Other mucosubstances on the surface of erythrocytes contain sialic acid, and the sialic acid residues of these substances have been identified as the receptors which bind influenza virus, giving rise to the phenomenon of viral hemagglutination. Incubation of the virus-agglutinated red blood cells for a time results in the release of the virus particles and sialic acid from the surface of the cells, and the erythrocytes lose their suscepti-

bility to viral agglutination. Release of the sialic acid is brought about by a viral enzyme which has been called "receptor-destroying enzyme" (25). Receptor-destroying enzymes (neuraminidases) have also been obtained from various bacteria (25). Mucosubstances which inhibit viral hemagglutination are found in serum and other body fluids and in all tissues (9). A glycoprotein with inhibitor activity has been isolated from the α_2-globulin fraction of human serum, and another hemagglutination inhibitor is associated with the α_1-fraction. The viral hemagglutination inhibitors are sialic acid-containing proteins, and treatment with active influenza virus or bacterial neuraminidases destroys their inhibitory capacity. All sialic acid-containing glycoproteins do not have inhibitor activity; orosomucoid is ineffective, even though the sialic acid of this glycoprotein is released by neuraminidase. The evidence indicates that the virus inhibitors may be similar to the mucosubstances on the surface of erythrocytes and that they inhibit viral hemagglutination by competing with the red blood cells for binding sites on the virus (9, 25, 26). The physiological significance of these inhibitors is not known.

Certain other plasma glycoproteins have been associated with specific physiological functions. Studies of these glycoproteins, although of a preliminary nature, have provided some insight into the problem of determining the types of roles played by the carbohydrate components of glycoproteins. Some of these glycoproteins are considered in the discussion to follow.

Some glycoproteins exhibit an ability to bind substances and are responsible for transporting these substances through the blood. Among these are the copper-binding protein, ceruloplasmin (25); the hemoglobin-

binding protein, haptoglobin (9); transferrin (siderophilin), which binds iron and other heavy metals (137); and the corticosteroid-binding protein, "transcortin" (9). Thyroid-binding protein also appears to be a glycoprotein, but this has not been definitely established (9). Of these, only in the case of "transcortin" has the carbohydrate component been implicated in the binding. Seal and Doe (138) have recently isolated "transcortin" in a highly purified form. The preparation was reported to contain 4 moles each of fucose and sialic acid per mole of protein. Treatment of the glycoprotein with neuraminidase split off sialic acid and resulted in the release of bound cortisol.

Other glycoproteins of particular interest are two components of the blood clotting system, prothrombin and fibrinogen (9, 25). Prothrombin contains hexose, hexosamine and sialic acid; fucose has not been reported. The conversion of prothrombin to thrombin is reported to result in a loss of 60 to 80% of the carbohydrate and only 40% of the total nitrogen. Pretreatment of beef prothrombin with neuraminidase, which renders about two-thirds of the sialic acid dialyzable, greatly increases the rate at which the prothrombin is converted to thrombin in citrate buffer, and it has been suggested that the carbohydrate of prothrombin is involved in maintaining the enzymatic inactivity of prothrombin (9). Fibrinogen contains galactose, mannose and hexosamine. Fucose is absent in the bovine protein and appears to be lacking in human fibrinogen (9). Proteolysis of fibrinogen by thrombin to form fibrin results in a relatively greater loss of carbohydrate than of peptide nitrogen, with little or no loss of hexosamine. No hexose has been demonstrated in any of the peptides produced during the conversion (9).

Considerable interest was aroused by a report that fetuin, a glycoprotein which occurs in large amounts in fetal and new-born calves, appeared to be the growth factor in fetal serum used for human tissue cultures. However, it has since been shown that this was not the case (139), and the role of this glycoprotein is still unknown.

Several of the protein hormones found in blood plasma are glycoproteins, and, in some instances, the carbohydrate appears to be essential for hormonal activity. All of the gonadotropins, both of pituitary and placental origin, which have been studied are glycoproteins (9). The hormonal activity of gonadotropin from pregnant mare's serum, which contains hexose, hexosamine, fucose and sialic acid, is destroyed by crude Taka-diastase and bacterial receptor-destroying enzyme (9). Brossmer and Walter (140) reported that treatment of human chorionic gonadotropin with neuraminidase resulted in release of N-acetylneuraminic acid and loss of activity. In addition to sialic acid, this hormone contains galactose, mannose, fucose and hexosamine. Recently, Gottschalk et al. (141) reported that when ovine follicle stimulating hormone was treated with a "highly purified" preparation of receptor-destroying enzyme, all of the sialic acid was released from the hormone and about 97% of the hormonal activity was lost. It was concluded that all of the sialic acid in the glycoprotein is terminal and that sialic acid is necessary for the activity of the hormone. Thyrotropin is also a glycoprotein (26), but there has been no report on the possible involvement of the carbohydrate component in the hormonal activity.

Sialic acid also appears to be essential to the activity of erythropoietin, a glycoprotein hormone which stimulates erythropoiesis. This

hormone also contains galactose, mannose, fucose, hexosamine and sialic acid. Treatment of a highly purified preparation with 0.005 N or 0.01 N HCl released sialic acid and destroyed the hormonal activity. A crude preparation of this hormone was also inactivated by treatment with active influenza virus.

The evidence related above, although based largely on studies with impure enzyme preparations, suggests that the carbohydrate components of glycoproteins may be involved in various types of activity. Although the above discussion has been limited largely to blood group substances and glycoproteins of plasma, glycoproteins are also found in solid tissues (142). The significance of these glycoproteins and many of those found in blood plasma is not clear.

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